Characterization of microbial community dynamics during anaerobic digestion of wheat distillery waste

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ABSTRACT

Anaerobic digestion of agricultural wastes provides an opportunity for renewable energy production while reducing emissions of greenhouse gasses such as CO_2 and CH_4 from crop and livestock production. While anaerobic digestion is possible under a wide range of temperatures and reactor configurations, it does require a stable methanogenic community composed of hydrolytic and fermentative bacteria and methanogenic archaea in order to maintain robust methane production.

Research focused on characterizing and optimizing the microbial community during anaerobic digestion is increasingly exploiting DNA-based methods. In addition to providing an in-depth phylogenetic survey, these techniques permit examination of dynamic changes in α- and β-diversity during the digestion process and in response to perturbations in the system. This study used universal target amplification, next generation sequencing, and quantitative PCR to characterize the Bacteria and Archaea in digestate from thermophilic batch anaerobic digesters processing different combinations wheat ethanol stillage waste and cattle manure. The results indicated that the bacterial community was composed primarily of Firmicutes, with Proteobacteria and Bacteroidetes also numerically abundant. While less phylogenetically diverse, the archaeal community showed robust populations of both hydrogenotrophic and acetoclastic methanogens. A core microbiome present across all reactors was identified and differences in the relative abundances of the bacteria within the core community suggested significant niche overlap and metabolic redundancy in the reactors.

A time-course study correlating the abundances of individual Bacteria and Archaea to methane production and volatile fatty acid catabolization identified several microorganisms hypothesized to be critical to both hydrogenotrophic and acetoclastic methanogenesis. Individual Bacteria most closely related to *Clostridium* spp. and *Acetivibrio* spp. were 10-1000-fold less abundant in reactors suffering from volatile fatty acid accumulation and inhibition of methanogenesis. Additionally, failing reactors were devoid of robust populations of acetoclastic methanogens.

Microorganisms identified as critical during the time-course study were targeted for isolation *in vitro* and a robust methanogenic consortium consisting of at least 9 bacteria and both a hydrogenotrophic and an acetoclastic methanogen was stably propagated. Addition of this bioaugmentation consortium to digesters experiencing classic symptoms of acid crisis resulted in reduced acetate accumulation and initiation of methanogenesis. One acetoclastic methanogen, most likely a novel species from the genus *Methanosarcina*, showed particularly robust growth in the recovered bioaugmented reactors, increasing 100-fold in the first 7 days post-treatment. A combination of Illumina shotgun and Roche 454 paired-end sequencing chemistry was used to generate a high quality draft genome for this organism. Analysis of the annotated genome revealed diverse metabolic potential with a full complement of genes for acetoclastic, hydrogenotrophic and methylotrophic methanogenesis pathways represented.

Taken as a whole, this thesis provides the foundation for using microbial community characterization to inform anaerobic digester design and operation. By identifying organisms of interest, correlating their abundance to specific biochemical functions and confirming their hypothesized functions *in situ*, microorganisms critical for robust

methane production were acquired. The logical extension of this work is to establish monitoring tools for microorganisms identified as critical to specific performance parameters, to enumerate them in real-time, and to use that data to improve reactor operation.

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CHAPTER 1 - Introduction and literature review

1.1 Anaerobic digestion of agricultural waste

The phenomenon that accumulated human and livestock waste generated combustible biogas as it decomposed has been documented since at least the 17th century, with John Dalton and Humphrey Davey confirming in the early 19th century that the flammable gas produced was methane (Abbasi 2012). Further study of this phenomenon by Bechamp (1868), Omelianski (1890), and Sohngen (1910) elucidated that the process relied on microorganisms, and that the underlying biochemical reactions were the oxidation of acetate and the redox reaction between carbon dioxide and hydrogen (McCarty 1982). The first documented industrial application of this technology was a design for a septic tank developed by Mouras in France constructed of brick and used for treating wastewater and sewage from his home. The septic tank design was further improved by Cameron in England and was so effective at reducing the volume of solids from municipal sewage waste that in 1897 the city of Exeter became the first to treat all of their wastewater using anaerobic digestion, with the biogas produced providing heat and light for the plant buildings (Pullen 2015). While the development of anaerobic digestion in Western Europe was primarily centered on waste disposal, it became an important source of renewable energy in developing countries early in the 20th century. India and China both instituted government-led programs to construct biogas plants that would use food and human waste to provide energy in rural areas. While fuel shortages during the Second World War spurred interest in anaerobic digestion for energy production, the low prices of fossil fuels throughout the second half of the 20th century curtailed its widespread adoption in the developed world (Abbasi 2012). More recently, the focus on

encouraging sustainable municipal and agricultural waste disposal practices has renewed interest in anaerobic digestion technologies.

The input material for anaerobic digestion can be any organic substrate however the process is uniquely well-suited to processing livestock and crop production waste as it reduces greenhouse gas emissions, improves sustainability and generates a value-added product for producers (Massé et al. 2011). In 2011, the agricultural sector generated 8% of total greenhouse gas emissions in Canada, of which 12% were directly related to manure storage from livestock production (Canada 2013). Incorporating anaerobic digestion as a manure management tool can reduce the viability of pathogens such as *Escherichia coli* and *Salmonella typhimurium* (Chen et al. 2012) and lessen the risk of contamination of crops and waterways. Additionally, digested manure has a significantly lower chemical oxygen demand (COD) compared to undigested manure, further reducing the effects of land application of manure on waterways (Massé et al. 1996). As an input material, manure has the added advantage of harboring a very diverse range of microbes permitting successful digestion under psychrophilic, mesophilic and thermophilic conditions using an array of reactor designs (Sakar et al. 2009; Weiland 2010). This microbial richness makes manure an excellent material for reactor amendment, and several types of agricultural and municipal wastes have shown more robust and stable methane production when co-digested with manure in comparison to mono-digestion (Macias-Corral et al. 2008; Westerholm et al. 2012a). In addition to manure, anaerobic digestion has shown some potential as a disposal method of whole animal carcasses, reducing environmental and odor concerns associated with burial or incineration (Franke-Whittle and Insam 2013).

Plant-based material has also shown high biochemical methane potential and anaerobic digestion is one option for adding value to non-food waste from crop production including leaves, stalks, and grain not suitable for human or livestock consumption. The complex structure of cellulosic or lignocellulose residues can require a more specialized bacterial consortium for efficient hydrolysis and often benefits from thermophilic operation which requires more energy input (Sawatdeenarunat et al. 2015). Anaerobic digestion does not contribute significantly to the breakdown of lignin, and applying biological pretreatment prior to digestion including heat, steam or de-lignifying microorganisms has been effective in increasing the biochemical methane potential of these residues (Frigon and Guiot 2010; Hendriks and Zeeman 2009). Despite these challenges, the energy potential from these residues using anaerobic digestion is comparable to lignocellulosic bioethanol production (Chandra et al. 2012).

The concept of coupling bioethanol production and anaerobic digestion to improve the efficiency of both systems has been explored over the last 30 years, using residues from corn (Lanting and Gross 1985), cassava (Zhang et al. 2010), and cereal grains (Mustafa et al. 2000) among others. The bioethanol plant provides a steady stream of high COD waste material while the energy produced by digesting the waste material provides additional green energy to offset inputs for ethanol production. The benefits include increasing overall energy production, reducing the carbon footprint and cost of bioethanol production, and reducing water consumption. Incorporating livestock manure into the biorefinery loop could provide further benefits by limiting the release of high organic content waste material into the environment while simultaneously producing renewable energy to offset energy used for both bioethanol and livestock production.

The wide-spread adoption of anaerobic digestion in the agricultural sector could substantially improve the sustainability of large scale-agricultural production, particularly in the livestock industry. In order to facilitate the incorporation of these systems into current production pipelines, anaerobic digestion must become more reliable, robust and cost-effective to operate (Massé et al. 2011).

1.2 Microbiology and biochemistry of anaerobic digestion

The conversion of organic waste into methane occurs in four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis and requires a complex microbial consortium of Bacteria and Archaea. Balance between the biochemical activities of these two groups is required to successfully catabolize organic waste material to methane.

To begin the process, hydrolytic bacteria break down the complex carbohydrates, proteins and lipids in the input material, converting them to more simple monomers and amino acids. Acidogenic bacteria then ferment these substrates into volatile fatty acids (VFA) with acetate, propionate, and butyrate typically the most abundant. The taxonomy of bacteria responsible for hydrolysis is dictated by the composition of the material. Digesters processing primarily cellulosic materials are most commonly dominated by *Clostridium* spp., with Bacteroidetes and Proteobacteria also commonly detected, although at lower abundance (Carballa et al. 2015). Lipid-rich materials also often contain abundant *Clostridium* spp. as well as other members of the phylum Firmicutes (Zakrzewski et al. 2012). *Clostridium* spp., *Bacillus* spp. and Proteobacteria are also associated with protein-rich substrates (Kovács et al. 2015; Solli et al. 2014).

Acetogenic Bacteria then convert propionate, butyrate, and formate to acetate via oxidation, producing H_2 and CO_2 in the process. These reactions are not energetically favorable, and in some cases, specific environmental conditions are required before they will proceed. The $\Delta G°'$ values for the oxidation of volatile fatty acids to form acetate are +48.1 kJ/mol and +76.1kJ/mol for butyrate and propionate respectively. The $\Delta G°'$ for the oxidation of acetate to form H_2 and CO_2 is +104.6 kJ/mol (Hattori 2008). These reactions require a very high concentration of volatile fatty acids, very low partial pressure of hydrogen, and a strongly reducing environment to facilitate interspecies hydrogen transfer (Hattori 2008). As a result, the organisms capable of volatile fatty acid catabolization rely on syntrophic relationships with acetogenic Bacteria and methanogenic Archaea to maintain these environmental conditions. Hydrogenotrophic Archaea scavenge the H_2 produced by these bacteria to produce methane, keeping the partial pressure of H_2 low, and enabling the growth of both syntrophic partners. If the digester is deficient in Bacteria and Archaea that can fill these metabolic niches, the result is inhibition of volatile fatty acid catabolization and absence of methane production. Members of the phylum Synergistetes have been associated with oxidation of acetate while *Syntrophomonas* spp. have been associated with catabolization of propionate and butyrate (Carballa et al. 2015). Identification and characterization of these syntrophic bacteria is difficult due to their specific culture requirements and slow growth rates.

All known methanogens are members of the taxonomic phylum Euryarchaeotes within the Domain Archaea. While there is a great deal of metabolic diversity within this taxonomic group, all methanogens produce methane during anaerobic metabolism.

Methanogens have been isolated from a range of environments including near thermal ocean vents and beneath Antarctic glaciers (Chaban et al. 2006). While they were originally thought to require strict anaerobic conditions and a very low redox potential for growth, members of the genus *Methanosarcina* have been shown recently to contain genes associated with aero-tolerance (Maeder et al. 2006). In addition to hydrogenotrophic and acetoclastic pathways, methanogenesis is possible via catabolization of methanol, formate and methylamines (Table 1-1) (Thauer et al. 1977).

Table 1-1 Biochemical reactions of methanogenesis.

		$\Delta G^{\circ\prime}$ (kJ/mol)
Hydrogenotrophic	$CO_2 + 4\ H_2 \rightarrow CH_4 + 2H_2O$	-130.7
Acetoclastic	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-32.3
Methanol	$4\ CH_3OH \rightarrow 2\ CH_4 + CO_2 + 2\ H_2O$	-79.9
Formate	$4\ HCOO^- + 4\ H^+ \rightarrow CH_4 + 3\ CO_2 + 2\ H_2O$	-36.1
Methylamine	$4\ CH_3NH_3^+ + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 4\ NH_4^+$	-57.4

H_2 and CO_2 produced via the oxidation of volatile fatty acids is catabolized to methane by hydrogenotrophic methanogens, of which *Methanoculleus*, *Methanobacterium*, and *Methanospirillum* are the most commonly found in mesophilic and thermophilic digesters (Nelson et al. 2011). Methanogenesis in these organisms can be inhibited by low sodium concentration which reduces methyltransferase activity. Additionally, they are often out-competed by faster-growing sulfate-reducing bacteria when the concentration of sulphate and nitrate are higher than 30 mM (Thauer et al. 2008).

Acetoclastic methanogens are capable of catabolizing acetate to generate methane directly, without the need for a bacterial partner. *Methanosaeta* spp. and *Methanosarcina* spp. are the most frequently observed in biogas-generating reactors (Nelson et al. 2011), although environmental conditions within the reactor often dictate their relative abundances. *Methanosaeta* are more readily inhibited by acetate concentrations above 100 mM, while *Methanosarcina* are more resistant to higher levels of volatile fatty acids, lower pH and ammonia accumulation (Hattori 2008). Additionally, *Methanosarcina* are more metabolically diverse and are capable of methanogenesis via hydrogenotrophic, acetoclastic and methylotrophic pathways (Maeder et al. 2006).

Identifying specific microorganisms associated with each biochemical stage of methanogenesis has been traditionally difficult due to their demanding culture requirements, and in the case of syntrophic organisms, generating a pure culture for *in vitro* characterization is almost impossible. Recent advancements in DNA sequencing technology have made large scale in-depth analysis of microbial communities from anaerobic digesters more feasible, providing much greater insight into the composition and dynamic changes in the reactors over the course of digestion and also highlighting

individual organisms that fill specific metabolic niches in the methanogenesis pathway. Temperature and input material have been identified as key drivers of differences in microbial community richness and diversity (Levén et al. 2007). In several cases, increased α-diversity, including species richness and evenness, has been found to benefit reactors with variable input concentration or loading rate, and those operating at thermophilic temperatures (Carballa et al. 2011; Schauer-Gimenez et al. 2010; Werner et al. 2011). Reactors amended with manure, compost, or other rich sources of microbial diversity, have been shown to have greater α-diversity and improved performance stability (Werner et al. 2011). The most common hypothesis is that the increased total metabolic capacity and the niche overlap present in reactors with greater microbial diversity are responsible for the stability in performance (Carballa et al. 2015) and that the loss of these characteristics can reduce the ability of the community to shift in response to changes in input material or operating conditions.

1.3 Microbial community profiling

Anaerobic digesters have been traditionally treated like a "black box", with operational controls based solely on the composition of the input and output material, and only minimal concern given to developing and maintaining the most efficient digester microbial community possible (Weiland 2010). Current research into increasing the output and capacity of anaerobic digesters is focused on microbial community characterization and monitoring (Vanwonterghem et al. 2014b).

Early scientific work characterizing the methane producing microorganisms from bogs and manure stockpiles relied purely on culture techniques. The challenges associated with isolating and propagating these organisms meant that while stable methanogenic

consortia were isolated *in vitro* as early as 1910, the first pure culture of a methanogen was not obtained until 1933 (Stephenson and Stickland 1933). Further refinements of culture methodology for strict anaerobes developed by Hungate and Balch led to the isolation and characterization of many more microorganisms from methanogenic communities, however the process was labor intensive and time-consuming (Balch et al. 1979; Hungate 1950) .

The development of the 16S rRNA universal target ushered in a new era of taxonomic classification of microorganisms based on DNA sequencing information (Woese and Fox 1977). This novel approach categorized methanogens as members of a new Archaeal Domain and these molecular tools became very useful for characterizing microbial communities from extreme environments. In addition to the commonly cited 16S rRNA, universal targets based on bacterial type I *cpn*60 (Hill et al. 2004) and archaeal type II thermosome chaperonins (Chaban and Hill 2012) have also been recently developed. These essential chaperone proteins facilitate protein folding and assembly and are highly conserved in both prokaryotes and eukaryotes (Hemmingsen et al. 1988). In the majority of microorganisms, *cpn*60 is also present as a single copy gene, facilitating enumeration of microorganisms using quantitative PCR (Hill et al. 2004).

The use of a protein-coding gene as a universal target permits *de novo* assembly of operational taxonomic units (OTU) as opposed to the clustering approach used for the aggregation and analysis of 16S metagenomic sequence data (Schloss and Handelsman 2005). OTU represent either individual or groups of microorganisms whose taxonomic relationship to each other is described based on the similarity of their DNA sequences. The use of OTU as a phylogenetic unit permits greater phylogenetic resolution of closely

related species (Links et al. 2012) and in contrast to 16S, the consensus sequence generated using OTU assembly is representative of all of the sequencing reads within that OTU. The result is a more accurate reference sequence for designing assays to enumerate individual microorganisms identified in the course of metagenomic studies.

Universal amplification has traditionally been followed by amplicon cloning, denaturing gradient gel electrophoresis (DGGE) or restriction fragment length polymorphism (RFLP) analysis. While sequencing clone libraries allows for greater phylogenetic resolution, the labor required for generating a clone library of sufficient size to generate a comprehensive phylogenetic survey or examine microbial community structure is prohibitive. By contrast, RFLP and DGGE produce an overall snapshot of the range of community members, but are not suitable for identifying changes at the species or subspecies level (Ercolini 2004; Ranjard et al. 2000).

Second generation sequencing platforms including pyrosequencing developed by Roche and high-throughput dye sequencing by Illumina, can generate millions of sequencing reads in a single run. These technologies enable characterization of a microbial community to a depth 10-100-fold greater than was previously feasible providing an in-depth phylogenetic survey and permitting a statistically robust examination using traditional ecological diversity metrics. Alpha-diversity comparisons include the number of species within a community (richness) as well as the relative abundances of individual species (evenness) for which the most common metrics are the Chao1 and the Simpson index (*D*) respectively. The Shannon index (*H'*) calculates the entropy in a given community and is influenced by both richness and evenness (Hill et al. 2003). While these metrics have introduced new statistical measures for comparing the structure of

microbial communities, it is important to consider the effect of library size when comparing α-diversity metrics between samples so as not to introduce bias (Gihring et al. 2012). Beta-diversity examines changes in microbial community composition and the phylogenetic relationship between different samples. Commonly deployed metrics include UniFrac (Lozupone and Knight 2005) and Bray-Curtis dissimilarity (Bray and Curtis 1957). While both metrics compare the number of shared species and their relative abundances, UniFrac also considers the phylogenetic distance between different members of the community and may provide more accurate comparison of communities containing clusters of phylogenetically similar organisms.

Applying these powerful tools to better understand the dynamic changes in the microbial community during anaerobic digestion, as well as provide a more complete survey of the bacteria and archaea critical to maximizing methanogenesis is one of the central goals of this thesis.

1.4 Anaerobic digester design and operation

Reactor design and operation are important considerations for maximizing process efficiency. Many different configurations have been used successfully, including incorporating single or multiple reactor cores, closed batch or continuous organic loading, and nutrient or biomass recycling (Weiland 2010). There are many factors to consider when implementing an anaerobic system for waste processing in order to maximize methane production and the breakdown of organic material while minimizing the retention or processing time required. The earliest designs for anaerobic digesters including the fixed dome, floating cover and balloon type reactors could be adapted for continuous flow or batch operation, required a long hydraulic retention time, and had no

active mixing of the material inside the reactor (Bond and Templeton 2011). As the microbial and biochemical dynamics were studied in greater detail, more complicated reactor designs were constructed to maximize the degradation of organic material and methane production.

Batch operation is the most simple reactor configuration as input material is loaded into the reactor to begin the process, the reactor is sealed, and biogas is continually siphoned off as it is produced. In this design, the biochemical steps of methanogenesis occur in sequence, beginning with an outgrowth of hydrolytic bacteria, followed by acidogenic and acetogenic bacteria, and finally methanogenic archaea. While this is more likely to result in the maximal production of each class of microorganism as they frequently consume their desired substrate until depletion, the retention time is often longer compared to other reactor designs. It is also difficult to accurately model batch digesters and predict their future performance as their inputs and microbial community compositions can undergo significant change between runs (Donoso-Bravo et al. 2011). As the start-up phase of the digestion process is often the most time-consuming and least productive part of the process, the fact that this step is repeated every time the reactor is loaded reduces system efficiency. However, the complete change-over in the reactor does allow for more flexibility in the amount and composition of the input being digested, without the concern of disrupting a steady state microbial community.

One of the other more common anaerobic reactor designs is a continuous flow system, with organic material continuously loaded, and biogas and digestate continuously removed. In this reactor design, all steps of the methanogenic pathway are happening concurrently. During the start-up phase of the system, the flow rate of organic material is

gradually increased, allowing for the microbial community to reach a steady state, whereby methane is produced and there is little to no accumulation of volatile fatty acids in the system indicating the activity of the acetogenic community is approximately equal to the metabolic activity of the methanogenic population (Donoso-Bravo et al. 2011). Once this steady state is achieved, the organic loading rate is constantly adjusted to maintain equilibrium between these two biochemical processes. Monod-type modeling of microorganism growth rates has been used to quantify and predict microbial community behavior in the reactor, although the requirement to base the model on the microorganism activity that is rate-limiting in the process may lead to misleading results if the community composition and efficiency at different stages undergoes any type of change (Donoso-Bravo et al. 2011). While this is a fairly simple reactor system to construct and operate, there are not many ways to easily adjust the system if performance begins to lag. Also, the operating conditions that are conducive to a reactor operating at steady state are likely not the maximal activity of the microorganisms present in the reactor, but a compromise for each so that neither is being completely inhibited.

To increase the stability of the microbial community in continuous-flow reactors, immobilization of the microbes has been examined. Up-flow anaerobic sludge blanket (UASB) reactors select for consortia that are naturally biofilm-forming, which develop into large sludge granules and form a suspended layer in the reactor. The use of synthetic materials as microbial supports have also been tested and found to reduce microbial washout (Ward et al. 2008). While these techniques can help retain microorganisms that may otherwise be lost during high flow-rate operation, the diversity of these digester

communities is lower, and may be less able to respond to variations in input material, temperature or pH (Nelson et al. 2012).

More recently, the need to try and optimize the different biochemical steps in the methanogenesis pathway has led to alternate reactor designs, specifically two-stage and plug flow digestion (Nasr et al. 2012). The two stage system typically features two continuous-flow reactors, one optimized for the hydrolytic and fermentative communities and the other for the methanogenic community. The organic input is continuously fed to the acetogenic reactor, with the high volatile fatty acid-containing output then transferred to the methanogenic reactor. This system enables maximum breakdown of the organic material as the acidified material can be loaded into the methanogenic reactor in a controlled way such that the volatile fatty acid concentration and pH are optimized for methanogenic activity. Research evaluating two-stage systems have shown an increase in total energy production of 5-18% when compared to digestion using a single continuous-flow system (Luo et al. 2011; Nasr et al. 2012).

The plug-flow reactor creates temporal separation between the fermentative and methanogenic stages similar to the batch digestion configuration, but also incorporates a continuous-flow component which allows for more flexibility during operation. While it often requires in higher hydraulic retention times compared to two stage digestion, total retention time is reduced compared to single tank batch digestion (Weiland 2010). The microbial succession pattern in this reactor design follows a similar time-course to batch digestion, with identifiable hydrolytic, acetogenic, and methanogenic community profiles distinguishable in different sections of the digester. This configuration is likely more conducive to achieving maximum bacterial and archaeal productivity as the reactor

design incorporates some level of physical distance and separation between the different metabolic functions in the methanogenesis pathway (Chung et al. 2013).

In additional to the flow of input material, level of mixing, and inoculation protocol, temperature is one of the most critical operation parameters during anaerobic digestion. While digesters of all configurations have been run successfully under psychrophilic (<25°C), mesophilic (25-55°C) and thermophilic (>55°C) conditions, the operational considerations with regard to maintaining microbial community stability for each situation are different.

Psychrophilic digestion has been possible when processing manure and while it requires almost no energy input, the amount of biogas produced trails mesophilic and thermophilic operations. The growth of methanogenic archaea is slower at temperatures cooler than 25°C, and significant acclimatization of the seed inoculum has been required to prevent a long lag phase in methane production during reactor start-up (Massé et al. 1996). Mesophilic digestion is the most common configuration, and has a reputation as being the most stable in term of consistent biogas production. Thermophilic digestion has the most potential to maximize biogas production, however it has been shown that maintaining an equilibrium between acidogenic and methanogenic activity is easier at mesophilic compared to thermophilic temperatures (Suryawanshi et al. 2010). Additionally, maintaining the reactor at thermophilic temperatures requires significantly more energy input into the system, decreasing the new energy gain of the reactor.

There are many operating variables to consider for maximizing methane production from anaerobic digestion with both input-specific and economic concerns informing reactor

design and operation. Greater insight into the dynamics of the microbial community during anaerobic digestion can be invaluable when considering reactor design and operation for maximizing waste consumption and methane production.

1.5 Dysbiosis in anaerobic digesters

Anaerobic digestion is dependent on complex biochemical pathways and requires balanced metabolic activity among several different organisms to maintain functionality. While the symptoms of digester dysbiosis have been well-documented, accumulation of ammonia and volatile fatty acids, decreased pH and low methane production, the underlying microbial causes of dysbiosis are still largely unknown. The most obvious diagnostic for dysbiosis is a reduction in the diversity and abundance of methanogens (Carballa et al. 2015). Most commonly, reactors that are robustly and consistently producing methane contain healthy populations of both acetoclastic and hydrogenotrophic methanogens. Acetoclastic methanogens are the most susceptible to environmental changes and a reduction in their number could be a signal of stress on the microbial community (Hattori 2008). In the bacterial community, there is such a diverse taxonomy of bacteria that have been associated with methane production from Archaea that is can be difficult to distinguish which are beneficial and which are harmful. Recent studies correlating specific bacteria to hydrolytic, acidogenic and acetogenic activities have helped to identify some known taxonomic groups that perform critical metabolic functions, as well as identify specific genes that are biomarkers for metabolic activity relevant to the methanogenic pathway. Culturing and studying these organisms i*n vitro* has also led to a wealth of knowledge about their growth requirements.

The root cause of reactor dysbiosis is often the inherent differences in growth preferences between the fermentative Bacteria and methanogenic Archaea. Hydrolytic and fermentative Bacteria typically have growth rates that are much faster than Archaea (Hattori 2008). Typically, there is also significantly more ecological richness in the bacterial community compared to the archaeal community. This level of metabolic diversity and niche redundancy naturally translates into a bacterial community that is much more efficient and productive than its archaeal counterpart. Any shift in the digester environment that shifts the archaeal community out of its ideal growth range will amplify any bacterial/archaeal imbalances.

Using this information, researchers are now trying to identify trends in microbial community composition and structure to more accurately describe healthy vs. unhealthy digester communities. Some results have shown that an increase in the abundances of certain taxonomic classes can be a sign of imbalance between the bacterial and archaeal metabolic activities. Of the acetoclastic methanogens, *Methanosaeta* have been shown to be the most susceptible to decreasing pH and ammonia accumulation, and a shift from *Methanosaeta* to *Methanosarcina* may be the first indication that conditions are deteriorating (Carballa et al. 2015). Digesters that have been running sub-optimally for some time, with prolonged periods of volatile fatty acid and/or ammonia accumulation are often completely devoid of these two genera and dominated by *Methanomicrobiales* and *Methanobacteriales* (Carballa et al. 2015). In the bacterial community, a shift from predominantly Firmicutes to predominately Proteobacteria has been linked with elevated VFA concentration and an increase in Bacteroidetes has been associated with high

concentrations of ammonia (De Vrieze et al. 2015), although it is unclear whether this shift is the cause or a symptom of worsening reactor conditions.

1.6 Bioaugmentation of anaerobic digesters

Bioaugmentation, the enhancement of the digester microbial community by adding individual or groups of microorganisms with desirable metabolic capabilities, has been associated with increased overall methane production and process stability (Schauer-Gimenez et al. 2010; Werner et al. 2011). Bioaugmentation has been tested in several forms, and may even begin prior loading input material into the reactor.

For very complex substrates, pre-treatment of the organic material prior to anaerobic digestion may facilitate the hydrolytic and acidogenic steps of the reaction. Plant material in particular contains a high percentage of both lignin and cellulose, which can be difficult for bacteria to access and break down. As a result, these systems can benefit immensely from a biological pre-treatment prior to digestion that renders these substrates more easily digestible. Fungi have been identified that are particularly adept at breaking down the complex structures of lignin and cellulose into acidic monomers and monosaccharides, and have been tested as biological pre-treatment options prior to anaerobic digestion. Fungal pre-treatment of lignocellulosic substrates has been shown to increase the biochemical methane potential of these substrates by facilitating hydrolysis and acidogenesis by bacteria (Chandra et al. 2012).

The most common method of bioaugmentation uses manure amendment in digesters processing plant or food production waste. Previous examination of the role of microbial community evenness and diversity has been linked to both reactor stability and

performance (Schauer-Gimenez et al. 2010; Werner et al. 2011). The ability to maintain a wide array of microorganisms in the reactor, with a very diverse range of metabolic properties, increases the probability of having an organism present to meet the changing requirements of a reactor environment that frequently changes along with input composition and loading rate. The most frequent way of maintaining reactor community richness is the addition of material rich in microorganisms, and manure is a readily available and commonly effective option. For several substrates, particularly those with a very high carbohydrate or volatile solids content, the addition of manure to the digester has resulted in increased performance stability and fewer incidents of methanogenic collapse (Labatut and Scott 2008; Westerholm et al. 2012a). Digestate recycling has also been examined as a way to continually maintain robust populations of critical microorganisms. Recycling of digestate from previous runs has been shown to improve performance, and cut down on lag time before the onset of methane production, especially for batch systems (Young et al. 2013). However, it is important to consider that constant recycling with consistent selective pressure for the most well-adapted microorganisms to the reactor community, may eventually lead to a reduction in richness, leaving the consortium less able to respond to any changes in input composition and operational parameters (Pervin et al. 2013).

The most targeted form of bioaugmentation is direct supplementation with microorganisms with desirable metabolic properties, and this type of reactor amendment is becoming more common as molecular methods are used to identify specific microorganisms associated with the biochemical steps of methanogenesis. The molecular information generated for these “high-performing” organisms can be used to inform

media conditions for their isolation and culture. Isolation and propagation *in vitro* will allow for a more precise determination of their growth requirements, with that knowledge then applied to reactor operation to increase production. This kind of experimental data for organisms targeted in this way has already identified species that may be of industrial value. In particular, *Methanosarcina* spp. have been shown to be very robust methanogens, with the ability to produce methane using both the hydrogenotrophic, acetoclastic, and methylotrophic pathways, a unique feature among methanogens (De Vrieze et al. 2012). This is an example of how the molecular examination of whole communities, followed by characterization of the most critical members of those communities, can lead to the development of molecular and biological tools for reactor monitoring and manipulation.

The availability of custom inoculum would provide a system recovery tool that could dramatically decrease lag time and boost performance and provide the opportunity for microbial intervention and manipulation that is currently not feasible. This could range from the addition of specific strains with desirable metabolic activities, with the express purpose of increasing that specific activity in the reactor, to amendment of the reactor with input rich in an array of microorganisms, such as manure. The addition of exogenous culture has been used successfully in a psychrophilic digester system, with the addition of a combination of syntrophic bacteria and hydrogenotrophic archaea increasing the total methane production by almost 50% (Akila and Chandra 2010). Not all exogenous culture amendments have been successful however, and the end result is likely dependent on the ability of the microorganisms to become established and proliferate within the experimental reactor environment (Fotidis et al. 2013).

Bioaugmentation of anaerobic digesters is still in its infancy, and the ability to manipulate microbial communities to achieve desired outcomes is still a work in progress.

OBJECTIVES

1. Characterize the bacterial and archaeal communities of thermophilic anaerobic digesters processing wheat-grain ethanol stillage waste and cattle manure.

2. Identify individual organisms whose numerical abundances correlate with digester production data including volatile fatty acid accumulation/catabolization and methane production.

3. Isolate and propagate microorganisms identified as having a correlation to digester production parameters, specifically acetate catabolization.

4. Examine the effect of bioaugmentation with isolated acetoclastic organisms on reactor recovery after a simulated organic overload event.

CHAPTER 2 - Microbial community composition is consistent across anaerobic digesters processing wheat-based fuel ethanol waste streams

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Citation

Town JR, Annand H, Pratt D, Dumonceaux TJ, Fonstad T. Microbial community composition is consistent across anaerobic digesters processing wheat-based fuel ethanol waste streams. Bioresource Technology. 2014;157(0).

Authors Contributions

Conceived and designed the experiments: JRT HA TJD TF. Performed the experiments: JRT HA DP. Analyzed the data: JRT HA TJD TF. Wrote the paper: JRT HA TJD TF.

2.2 Abstract

Biochemical methane potential (BMP) assays were conducted on byproducts from dry-grind wheat-based ethanol plants amended with feedlot manure at two input ratios. Whole stillage (WST), thin stillage (TST) and wet cake (WCK) were tested alone and with 1:1 and 2:1 ratios (VS basis) of byproduct:feedlot manure in bench-scale batch reactors. The addition of manure increased both the rate and consistency of methane production in triplicate reactors. In addition, digesters co-digesting thin stillage and cattle manure at 1:1 and 2:1 stillage:manure produced 125% and 119% methane expected based on the biochemical methane potential of each substrate digested individually. Bacterial community analysis using universal target amplification and pyrosequencing indicated there was a numerically dominant core of 42 bacteria that was universally present in the reactors regardless of input material. A smaller-scale analysis of the archaeal community showed that both hydrogenotrophic and acetoclastic methanogens were present in significant quantities.

2.3 Introduction

Global bioethanol production has increased in recent years, due to environmental pressures, and is the most common renewable biofuel for motor vehicles (Sarkar et al. 2012). In Canada, fuel ethanol production is dominated by the fermentation and distillation of starchy grains like corn and wheat. For every liter of ethanol produced via grain fermentation, between 8 and 15 liters of byproduct effluent is generated and must be disposed of (Saha et al. 2005). In a plant producing corn-based ethanol, downstream processing of these waste streams consumed 46.8% of the plant's total energy needs (Eskicioglu et al. 2011). Disposal of these waste streams can be major economic

limitation to ethanol production, negatively impacting the financial feasibility and energy balance of ethanol facilities. Bottlenecks in downstream waste processing can also disrupt system balances and delay further ethanol production. The whole stillage waste generated during ethanol production can be further separated into its liquid (thin stillage) and solid (wetcake) components using centrifugation. Research into disposal methods for this waste stream have primarily focused on processing thin stillage due to its high chemical oxygen demand (COD) and environmental impact on both soil and waterways.

Anaerobic digestion of ethanol byproducts could potentially provide a disposal method for bioethanol waste, while returning both heat and electricity to the process. The concept of a closed loop biorefinery system, where the byproduct of one entity becomes input for the next, links these processes with the overall system operating in concert. The result would be a decrease in the carbon footprint of the bioethanol facility, and an improved net energy balance for bioethanol production. The engineering and economic challenges of integrating ethanol production and anaerobic digestion have already been examined for corn stover and sugarcane (Liska et al. 2009; Rabelo et al. 2011) but the methane generating potential of wheat-based ethanol byproducts has not been widely published.

Co-locating an ethanol plant and an anaerobic digester at a beef feedlot could provide even more economic and environmental advantages. Manure is a widely used feedstock for anaerobic digestion because it decreases the volume of greenhouse gas emissions released during normal manure storage (Møller et al. 2004). Manure itself is a good substrate for co-digestion with other organic material because it can adjust the carbon-to-

nitrogen (C:N) ratio of feedstock, provide buffering capacity, and supply essential nutrients that improve methane yields (Labatut and Scott 2008; Ward et al. 2008). The biogas potential of manure is highly variable and it depends on the type of animal, the animal's feed, climate conditions, the type of bedding used, and the storage conditions of manure before anaerobic digestion occurs (Møller et al. 2004).

Co-digestion of feedlot manure with ethanol byproducts has been shown previously to increase both methane yield and process stability during the digestion of agricultural and ethanol production wastes (Westerholm 2012; Ye et al. 2013). The increased richness of microorganisms and nutrients achieved using manure amendment has also been shown to increase the stability of the process while improving the ability of the community to respond to operational changes and toxin exposure (Schauer-Gimenez et al. 2010; Werner et al. 2011).

A more thorough examination of the biochemical methane potential of these substrates, both singly and in combination, will help to determine the economic feasibility of this biorefinery model. This also provides an opportunity to characterize the microbial communities present in thermophilic digesters processing ethanol byproduct waste. Current knowledge gaps in this area, particularly with regards to the digestion of wheat-based ethanol byproducts, make it difficult to ensure optimal reactor design and operational conditions to achieve the maximum methane potential for these substrates. Next generation sequencing technologies provides an opportunity to characterize the bacterial and archaeal communities of these digesters and identify attributes of both

community structure and composition that contribute to methane production in this system.

2.4 Materials and Methods

Input materials

Ethanol byproducts were sampled from Terra Grain Fuels (Moose Jaw, SK, Canada), a dry-grind wheat-based ethanol plant. Samples were collected and then stored at 4°C until needed. Manure samples were collected from an Alberta beef feedlot for the 1:1 trial and from a Saskatchewan beef feedlot for the 2:1 trial and stored at 4°C until required. Seed inoculum was obtained from a HiMark Biogas anaerobic digester (Vegreville, AB, Canada) , operating primarily on feedlot manure, stored at -20°C, and used for both manure amendment trials. Prior to the start of each trial, the inoculum was thawed and incubated in a sealed bench-scale reactor containing an N_2 atmosphere at 55 ± 2°C for 5 and 7 days respectively. TS and VS were determined by standard methods (APHA 1995) with a modified incubation temperature of 70°C during TS determination to prevent loss of volatile solids (Angelidaki et al. 2009). TS, VS and VS/TS ratio for ethanol byproducts, manures and inoculum used in each experiment are outlined in Table 2-1.

Table 2-1 Characterization of wheat ethanol byproducts, manure and inoculum (n=3). a Initial TS and VS measurements were used for both trials.

EtOH byproduct: Manure	Substrate	% TS	% VS	% VS/TS
1:1	WST	17.68 ± 0.75	16.26 ± 0.73	91.94 ± 5.66
	TST	15.79 ± 0.03	14.07 ± 0.46	89.08 ± 2.90
	WCK	32.45 ± 0.44	31.37 ± 0.42	96.69 ± 1.83
	MAN	32.59 ± 4.46	23.63 ± 3.48	72.51 ± 14.57
2:1	WST	19.13 ± 1.31	17.72 ± 1.42	92.62 ± 9.77
	TST	13.33 ± 0.11	11.77 ± 0.41	88.29 ± 3.14
	WCK	34.07 ± 0.16	33.01 ± 0.17	96.89 ± 0.67
	MAN	38.66 ± 3.16	17.53 ± 0.86	45.34 ± 4.33
	INC[a]	9.42	6.43	68.25

Biochemical methane potential (BMP) assay

Two BMP experiments were performed to determine the ultimate methane yield and methane production rate that could be achieved from ethanol byproducts receiving two different ratios of feedlot manure. BMP assays were performed under thermophilic (55 ± 2°C) conditions as described previously (Angelidaki 2009; Owen et al. 1979). Ethanol byproduct was combined with feedlot manure in a 1:1 or 2:1 ratio of byproduct:manure based on VS content. This mixture was then combined 1:1 with inoculum. The volume of input material was adjusted to 5% TS in 300 ml with sterile water. Each input combination was incubated in triplicate 1 L bench-scale reactors sealed with screw caps fitted with rubber septae. Samples were taken to measure actual TS, VS and pH of each prepared mixture (Table 2-1). The headspace of the sealed bottles was flushed with N_2 gas for 5 minutes at room temperature, bled down to 3.45 kPa (0.5 psi) and incubated at 55±2ºC. Biogas accumulation was assayed using a pressure transducer equipped with a 25G sampling needle and pressure readings were converted to biogas volumes at standard temperature and pressure. Gas samples were taken using a 20ml syringe equipped with a stopcock and 25G needle, transferred to a dehumidified, evacuated 5 ml vial, and stored at 4ºC until analysis. After sampling, the bottles were vented down to 3.45 kPa (0.5 psi), swirled gently, and returned to the incubator. The experiment concluded when the daily biogas production volume dropped below 1% of the total accumulated biogas for each trial; day 38 for trial 1 and day 42 for trial 2.

Table 2-2 Average methane yield, methane production rate, and digestate pH in triplicate reactors processing whole stillage (WST), thin stillage (TST) or wetcake (WCK), with manure in a 1:1 or 2:1 ratio of byproduct:manure (WST-M, TST-M, WCK-M).

			CH_4 yield (B_o)	CH_4 production rate		pH	
		Substrate	**(ml/g VS added)**	***k* (day^{-1})**	**R^2**	**Initial**	**Final**
Trial 1	**Unamended**	WST	578 ± 14	0.094	0.927	7.76	7.81
		TST	483 ± 59	0.058	0.821	7.37	7.89
		WCK	493 ± 32	0.102	0.987	7.75	7.66
	1:1 Byproducts: Manure	WSM	389 ± 15	0.113	0.980	7.97	7.59
		TSM	446 ± 12	0.110	0.989	7.67	7.64
		WCM	344 ± 12	0.115	0.958	7.97	7.55
		MAN	230 ± 16	0.104	0.915	8.19	7.45
	Control	INC	147 ± 4	NA	NA	8.01	7.87
Trial 2	**Unamended**	WST	533 ± 18	0.106	0.977	7.41	7.70
		TST	592 ± 37	0.090	0.983	7.31	7.78
		WCK	485 ± 19	0.105	0.990	7.58	7.57
	2:1 Byproducts: Manure	WSM	399 ± 18	0.109	0.995	7.68	7.59
		TSM	523 ± 13	0.110	0.988	7.71	7.66
		WCM	367 ± 12	0.105	0.990	7.81	7.44
		MAN	136 ± 12	0.102	0.933	8.18	7.42
	Control	INC	120 ± 2	NA	NA	8.03	7.84

Biogas Composition

Biogas samples were analyzed using gas chromatography (Angelidaki et al. 2009). The relative percentages of CH_4, CO_2, H_2, N_2 and O_2 were determined using a Varion model 450-GC with front and middle TCD detectors (CP-2003, Agilent, Santa Clara, CA, USA). Injector, oven and detector temperatures were 100°C, 50°C and 150°C, respectively. The front column was a Hayesep Q 80/100 CP81069 (1 m x 3.175 mm) using argon make up gas flowing at 20 ml/min. The middle column was a Molsieve 5A 80/100 CP81025 (1 m x 3.175 mm) using helium make up gas flowing at 20 ml/min. The standard gas used for calibrating the GC was composed of H_2 (0.5%), CH_4 (40%), N_2 (1%), O_2 (5%), CO_2 (bal%).

Biogas yields were corrected to account for endogenous metabolism of the inoculum by subtracting the average biogas produced in the INC control reactors and reported as CH_4/g VS added (Angelidaki 2009). Expected biogas yields (B_o) for manure amended reactors were calculated by adding the proportional biogas production from the ethanol byproduct (EB) and manure (MAN) mono-digestions (Ye et al. 2013) using Equation 2-1 and Equation 2-2 below:

Equation 2-1 Expected biogas yield for manure amended reactors in Trial 1.

$$B_{o\text{Expected}} = \frac{1}{2} B_{o\text{EB,mono}} + \frac{1}{2} B_{o\text{MAN,mono}}$$

Equation 2-2 Expected biogas yield for manure amended reactors in Trial 2.

$$B_{o\text{Expected}} = \frac{2}{3} B_{o\text{EB,mono}} + \frac{1}{3} B_{o\text{MAN,mono}}$$

In addition to calculating the mean accumulated methane yield (B_o) across replicates in each trial, data points from the methane production profiles were plotted according to Equation 2-3 and used to determine the methane production rate (k). Regression analysis was used to describe the fit of the data to first-order rate kinetics (Angelidaki 2009):

Equation 2-3 Methane production rate

$$ln\frac{(B_G - B)}{B_o} = -kt$$

Universal target amplification and sequencing

Total genomic DNA was extracted from all end product digestate samples from triplicate reactors using a modified bead beating method as described previously (Dumonceaux et al. 2006). The bacterial *cpn*60 (type I chaperonin) universal targets were amplified using multiplex identifier (MID) tagged primer cocktails and amplification conditions previously shown to maximize the range of organisms detected (Hill et al. 2005). Briefly, each reaction contained: 1X PCR buffer (Invitrogen, Carlsbad, CA, USA), 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 100 nM each of H279/H280, 300 nM each of H1612/H1613 and 1U of Platinum Taq (Invitrogen, Carlsbad, CA, USA) and cycling conditions of 1 x95°C, 5 min; 40x 95°C 30sec, 42-60°C 30sec, 72°C 30sec; 1x 72° 2min. Amplification products from triplicate digesters were pooled and then sequenced using the Roche 454 GS FLX with Titanium chemistry (Branford, CT, USA).

Type II chaperonin (archaeal thermosome) targets were amplified using the same reaction mixture with 437.5 nM each of JH0175/JH178 and 62.5 nM each of JH0268/JH0269 and cycling conditions of 1 x95°C, 5 min; 40x 95°C 30sec, 55°C 30sec, 72°C 30sec; 1x 72° 2min. Amplicon from all digestate was pooled and used in a shotgun cloning reaction

with pGEM-T Easy vector (Promega, Madison, WI, USA). Clones were sequenced using Sanger chemistry, generating 181 sequencing reads.

Sequencing data analysis

Pyrosequencing reads were processed using mPUMA (Links et al. 2013) and assembled into distinct operational taxonomic units (OTU) using Trinity with an Inchworm kmer size of 31bp. OTU frequency data for each sequencing library was calculated using a data set of 12,757 randomly subsampled pyrosequencing reads; corresponding to the size of the smallest library. OTU frequency data was used as input for Mothur (Schloss et al. 2009) to calculate microbial community diversity. Principal coordinate analysis (PCoA) was done using Unifrac (Lozupone et al. 2006) to examine the phylogenetic similarity between reactors. Clone sequences were cleaned and trimmed using LUCY (Chou and Holmes 2001) and assembled into distinct OTU using CAP3 (Huang and Madan 1999).

Quantitative PCR assays

OTU-specific primers were designed using Beacon Designer v 7.0 (Premier Biosoft, Palo Alto, CA, USA). Quantitative PCR standards (10^1-10^7 gene copies/reaction) were generated using cloned amplicon. Target OTU sequences were amplified using EvaGreen qPCR amplification mix (BioRad, Hercules, CA, USA) with 400 nM of each primer. Primer sequences and amplification conditions are outlined in Table 2-3.

Table 2-3 OTU-specific qPCR primer sequences and amplification conditions.

Primer Name	Primer Sequence (5'-3')	Amplification conditions	Target
D0299	CCTAAGCGTTCCCATAGAA	95°C 5 min (1x)	
D0300	TTGCCTCTTCCTGGTCTA	95°C 15 sec; 55°C 20sec; 72°C 20 sec (40x) 72°C 2 min (1x)	OTU795
D0226	GGCCAAGGAAATACTCAA	95°C 5 min (1x)	
D0227	CTTGCCTTTTCTGTACCC	95°C 15 sec; 61°C 20sec; 72°C 20 sec (40x) 72°C 2 min (1x)	OTU501

2.5 Results and Discussion

Biogas production

When digested alone, the biogas potential of the ethanol byproducts was consistent between the two trials and there was no significant difference in mean methane accumulation between reactors processing thin stillage, whole stillage or wetcake (α=0.01). Manure amendment resulted in lower overall methane accumulation in reactors processing whole stillage in both trials, while manure amended reactors processing wetcake showed significantly less methane accumulation only at the 2:1 ethanol byproduct: manure (α=0.01). Mean methane accumulation for reactors processing thin stillage did not differ significantly from their manure amended counterparts (α=0.01), despite the lower methane potential of manure (Møller et al. 2004), and the reduced amount of ethanol byproduct available in the digester.

Manure-amended digesters also had more consistent methane production when compared to their unamended counterparts, resulting in reduced standard deviation for methane accumulation among triplicate reactors Table 2-2. This was particularly true in the case of thin stillage, which was highly variable in Trial 1. Additionally, the rate of methane production (k) increased with manure amendment for all of the byproduct streams, with a corresponding improvement of the fit of first order kinetics (R^2) for methane accumulation Table 2-2. This suggests a manure amended reactor system could provide the necessary stability for an operation processing primarily wheat-based ethanol production waste to function successfully, as previous studies have shown reactors processing stillage as the sole substrate have shown highly variable production and even complete collapse of methanogenesis (Westerholm 2012; Ye et al. 2013) over the long

term. As well, the increased rate in production could translate into a shorter hydraulic retention time, enhancing the waste processing capacity of the digester.

Manure amendment had differential effects on the three ethanol byproduct waste streams evaluated. When comparing the observed vs. expected methane accumulated in the manure amended reactors, whole stillage and wetcake amended with manure at 1:1 ethanol byproduct:manure produced 95% and 96% of the expected methane based on the BMP of the byproducts alone Table 2-4. When the products were amended with manure at 2:1, methane production for both whole stillage and wetcake was 100% of the expected volume Table 2-4. Reactors processing thin stillage amended with manure in both a 1:1 and 2:1 produced 125% and 119% of expected methane respectively, suggesting a synergistic reaction between the two substrates, with the combination of manure and thin stillage producing more methane then either input in isolation Table 2-4.

Table 2-4 Average observed and expected methane accumulation in triplicate digesters processing whole stillage (WST), thin stillage (TST) or wetcake (WCK), with manure in a 1:1 or 2:1 ratio of byproduct:manure (WST-M, TST-M, WCK-M).

		Methane yield, B_o (ml/g VS added)			
	Substrate	**Observed**	**Expected**	**% of Observed**	**% of Unamended**
1:1	**WSM**	389 ± 15	404 ± 21	96%	67%
	TSM	446 ± 12	357 ± 35	**125%**	**92%**
	WCM	344 ± 12	362 ± 24	95%	70%
2:1	**WSM**	399 ± 18	401 ± 23	100%	75%
	TSM	523 ± 13	440 ± 32	**119%**	**88%**
	WCM	367 ± 12	369 ± 22	100%	76%

Type I (bacterial cpn60) community analysis

The pyrosequencing libraries assembled into 1172 total OTU. The bacterial communities of the end product digestate were very similar in composition, despite the range of input combinations tested and the addition of manure from different geographic locations. The most frequently detected OTU were most closely related to members of the phylum Firmicutes, although they had relatively low sequence similarities to known organisms, making a precise taxonomic identification difficult (Figure 2-1). Previous studies examining the bacterial composition of digesters processing similar waste streams have shown organisms from both Clostridia and Bacteroidetes to be highly abundant (Ziganshin et al. 2013). Quantification of individual organisms closely related to *Clostridium* spp. have also been linked to reactor performance measures, specifically methane production, volatile fatty acid production, and VFA catabolization (Town et al. 2014b).

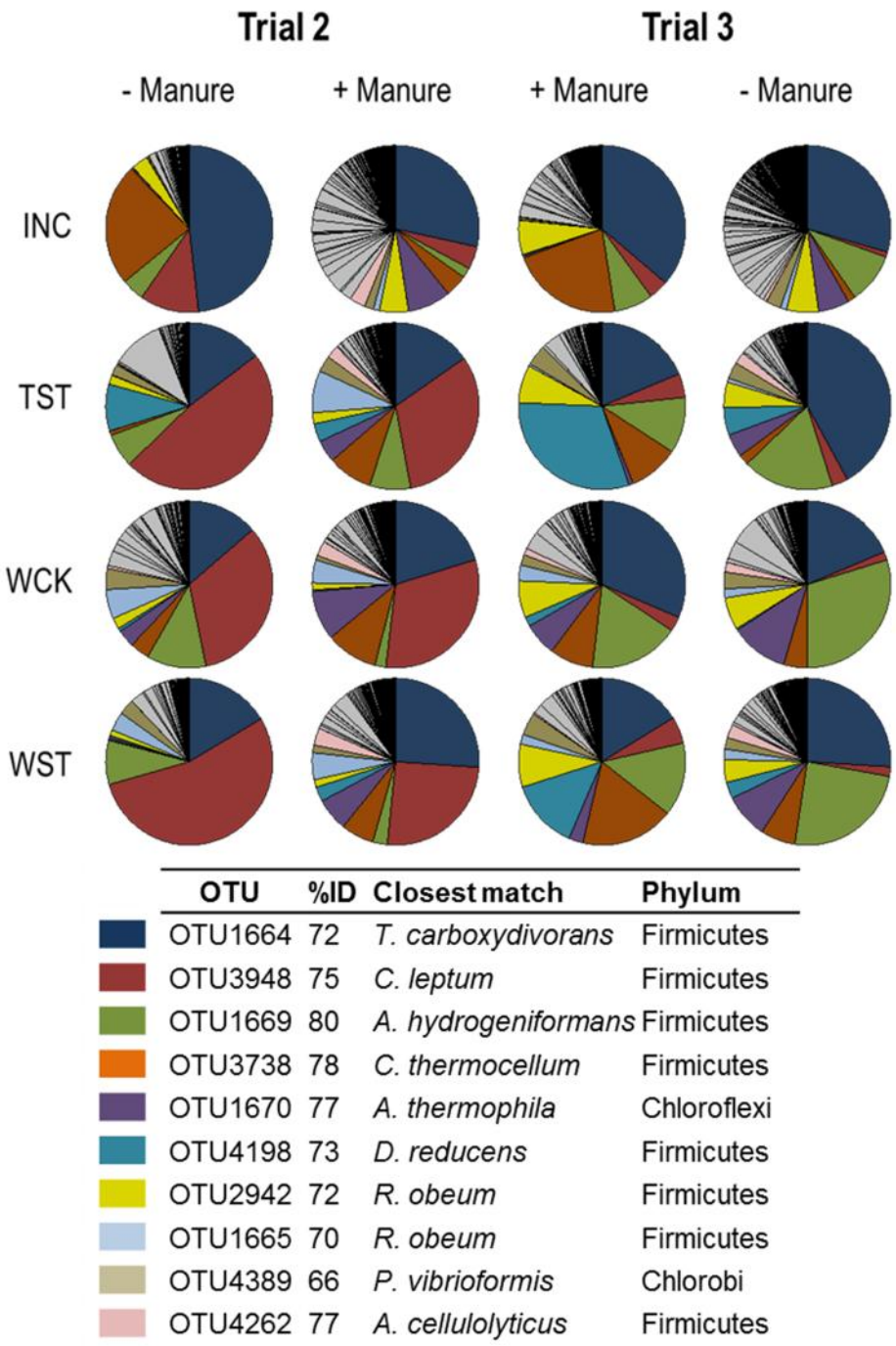

Figure 2-1 Pyrosequencing reads were generated using pooled type I chaperonin (*cpn*60) amplicon from triplicate digesters processing inoculum (INC), thin stillage (TST), wetcake (WCK) or whole stillage (WST) either alone or in combination with manure at a 1:1 (Trial 1) or 2:1 (Trial 2) ratio of ethanol byproduct:manure. Reads were assembled using mPUMA, and the relative abundances of individual OTU are shown.

Principal component analysis (PCoA) of unweighted Unifrac values showed very little intra- or inter-trial variation, suggesting that the bacterial community composition was very similar in all reactors regardless of input (Figure 2-2). By contrast, PCoA of weighted Unifrac values showed a comparatively strong clustering of samples by trial, indicating that while the presence or absence of OTU was similar between the two trials, there were differences in the relative abundances of those OTU (Figure 2-2). These results likely reflect the strong selective pressure present in this environment, with only certain organisms able to proliferate consistently. Given that there were no significant differences in the methane production from each input combination between the two trials, the difference in the relative abundances of these organisms between trials may suggest that there is some degeneracy in the community, with multiple microorganisms able to fulfill a given metabolic niche.

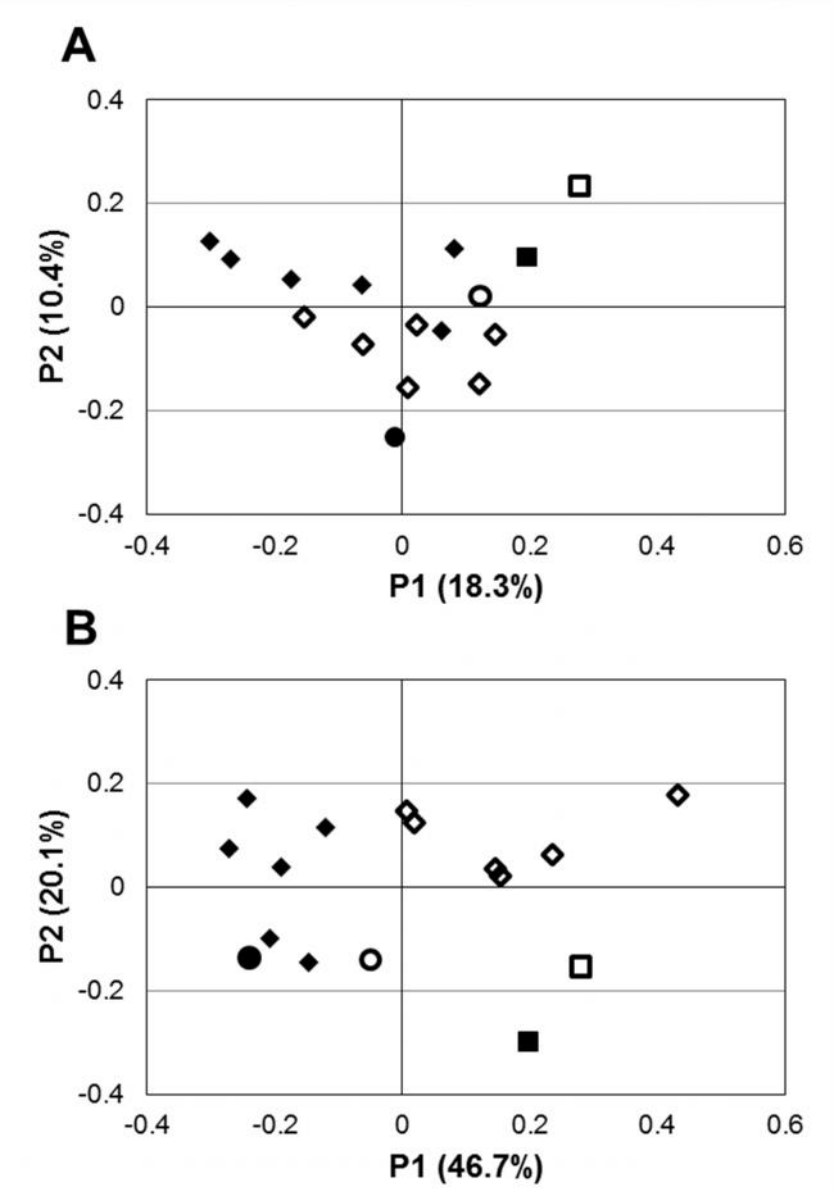

Figure 2-2 Subsampled OTU-frequency and phylogenetic data was used as input for Principal Component Analysis (PCoA) of unweighted (A) and weighted (B) Unifrac values for Trial 1 (closed) and Trial 2 (open) reactors processing ethanol byproducts +/- manure (diamond), inoculum alone (circle) or manure alone (square). The Unifrac metric is a measure of the phylogenetic distance between two complex microbial communities and may be calculated based solely on community composition (unweighted), or reflect composition and relative OTU abundances (weighted) (Lozupone et al. 2006). The axes represent the percent variation explained with the first two principal components (P1 and P2).

Manure amendment, which provides a rich source of both micronutrients and microorganisms, was associated with an increase in bacterial community diversity in the end product digestate (Figure 2-3). Given that only end point samples were tested, it is difficult to statistically correlate the increase in diversity to the increase in stability seen in the manure amended reactors compared to their unamended counterparts. However, this phenomenon has been seen previously, with digesters containing the most rich and diverse group of microorganisms able to respond more quickly to operational changes and toxin exposure (Schauer-Gimenez et al. 2010; Werner et al. 2011).

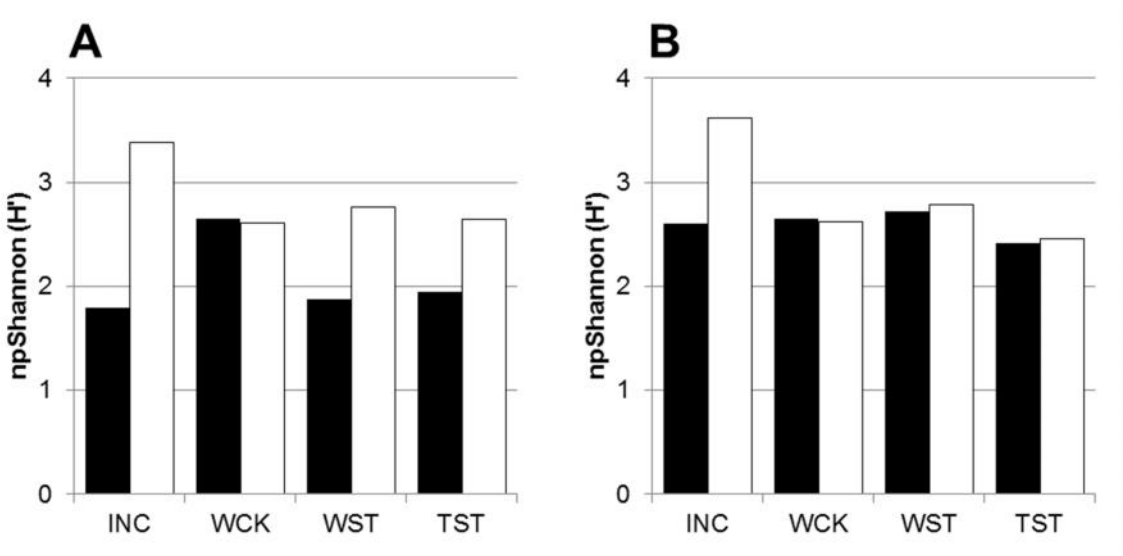

Figure 2-3 Bacterial community diversity in end-product digestate from reactors processing inoculum (INC), thin stillage (TST), wetcake (WCK) or whole stillage (WST) either alone (black) or in combination with manure (white) at a 1:1 (A) or 2:1 (B) ratio of ethanol byproduct:manure. OTU frequency data (subsampled to a consistent library size) was used as input to calculate Shannon's diversity index (*H'*) using Mothur.

An analysis of the OTU-frequency data also revealed a core of 42 OTU that appeared in every library in both trials, regardless of the input material in the digester (Table 2-5). Reads assigned to this core accounted for 92% and 95% of the total pyrosequencing reads in Trial 1 and Trial 2 respectively, indicating that these organisms consistently came to dominate the community by the end of the digestion period. A phylogenetic analysis of the core community revealed five broad groups of organisms, with the largest proportion likely members of the phylum Firmicutes; OTU similar to members of the phyla Proteobacteria and Actinobacteria were also identified (Figure 2-4). The relative abundances of the core OTU between the two trials also suggested that there were only a few OTU that accounted for the clustering pattern seen in the PCoA analysis of weighted Unifrac values, with the majority of core OTU present in similar abundance in both trials (Figure 4). Many of these OTU did not have significant matches in the cpnDB reference database (<80% sequence identity), making a precise taxonomic analysis difficult and highlighting the need for more research into the microorganisms that are able to flourish in this highly selective environment.

Table 2-5 Frequency table for the 42 core *cpn*60 OTU found in pyrosequencing libraries from all reactors.

			Trial 1								Trial 2							
OTU	**% ID**	**Nearest Neighbor**	**INC**	**MAN**	**TST**	**TST-M**	**WST**	**WST-M**	**WCK**	**WCK-M**	**INC**	**MAN**	**TST**	**TST-M**	**WST**	**WST-M**	**WCK**	**WCK-M**
4319	98.1	*Corynebacterium efficiens*	4	34	2	23	2	16	1	19	21	7	4	7	2	4	5	2
438902	88.6	*Aminobacter aminovorans*	53	172	24	70	8	76	19	91	148	286	25	95	38	111	59	80
430723	86.6	*Propionibacterium acidipropionici*	5	219	2	59	1	390	4	256	18	60	3	18	5	7	6	1
4346	86.5	*Pelagibacterium halotolerans*	14	22	5	7	1	12	3	12	26	42	5	12	13	11	6	5
4250	85.8	*Roseovarius nubinhibens*	22	299	10	18	3	41	11	75	28	139	20	30	12	26	14	21
4384	85.4	*Pelagibacterium halotolerans*	5	7	3	7	5	9	11	9	14	21	7	3	2	8	1	4
4367	85.1	*Corynebacterium halotolerans*	6	64	2	13	1	21	4	21	11	221	1	9	7	25	7	7
4281	83.6	*Pelagibacterium halotolerans*	2	4	1	2	1	2	2	4	9	17	2	1	3	5	2	2
4090	81.9	*Clostridium stercorarium*	111	255	53	164	62	187	235	131	132	134	35	79	36	51	71	34
430722	81.6	*Oceanibaculum indicum*	22	19	6	27	11	34	19	24	111	84	23	31	11	43	30	19
4392	81.3	*Clavibacter michiganensis*	19	75	4	39	5	58	11	56	48	146	11	29	8	42	12	10
4388	80.5	*Arthrobacter sp.*	13	91	2	22	1	24	12	26	47	53	8	26	10	12	15	13
4235	80.1	*Pelotomaculum thermopropionicum*	31	35	79	19	7	34	2	20	203	103	29	32	118	49	153	70
1669	79.7	*Anaerobaculum hydrogeniformans*	559	247	827	999	1,081	355	1,455	271	950	1,243	1,374	2,242	1,799	3,108	2,215	3,769
3738	78.4	*Clostridium thermocellum*	2,995	540	104	1,095	18	804	476	1,269	2,756	188	1,227	255	2,289	855	1,073	566
4160	77.7	*Clostridium thermocellum*	7	1	1	4	3	7	2	3	6	1	6	4	4	4	3	1
1670	77	*Anaerolinea thermophila*	50	1,051	5	542	53	806	430	1,206	48	759	104	571	351	1,096	815	1,418
4265	75.7	*Desulfotomaculum kuznetsovii*	2	2	1	3	7	8	9	1	23	8	5	9	7	14	1	1
4147	75.5	*Anaerotruncus colihominis*	9	37	38	12	8	14	23	13	79	238	38	157	45	183	96	174
3948	75.1	*Clostridium sp.*	1,447	556	6,067	4,004	6,832	3,218	4,179	3,994	480	121	586	395	659	234	362	199
423322	74.6	*Heliobacterium modesticaldum*	2	19	4	7	2	46	11	12	9	3	3	16	12	10	13	6
4233	74.5	*Heliobacterium modesticaldum*	11	204	5	27	7	155	57	63	67	37	48	122	89	118	77	55
4231	74.2	*Caldalkalibacillus thermarum*	2	1	5	8	23	20	18	1	6	5	71	28	79	99	8	6
4386	73.6	*Staphylococcus saprophyticus*	9	10	1	10	2	6	4	2	11	10	3	4	5	10	2	2
4197	73.6	*Tepidanaerobacter sp.*	22	294	1	26	8	48	24	75	12	231	7	22	11	30	15	67
1664	73.3	*Acetonema longum*	6,099	3,577	1,856	1,927	2,073	3,284	1,725	2,551	4,573	3,730	2,363	5,323	2,053	3,289	3,987	2,359
4198	73	*Desulfotomaculum reducens*	5	2	1,149	446	51	358	85	38	31	1	3,914	686	1,723	405	234	42
4376	72.7	*Thermus igniterrae*	6	190	31	11	18	29	67	16	8	28	8	16	9	10	30	8
2942	72.5	*Desulfotomaculum carboxydivorans*	412	704	227	270	139	187	271	171	847	784	931	616	1,053	557	907	793
4144	72.2	*Anaerotruncus colihominis*	6	35	6	16	7	23	10	14	13	49	28	37	12	35	10	15
423323	70.1	*Desulfobacter postgatei*	14	101	50	16	30	19	94	20	13	21	15	5	19	11	19	5
4288	69.7	*Porphyromonas gingivicanis*	7	49	17	86	45	52	196	16	38	143	413	200	359	203	333	433
1665	69.4	*Desulfotomaculum hydrothermale*	10	152	13	1,030	463	656	721	566	9	150	57	100	244	262	385	226
4205	67.1	*Caldalkalibacillus thermarum*	14	19	2	14	13	13	49	9	8	18	2	8	15	14	43	64
438901	66.4	*Prosthecochloris vibrioformis*	43	220	265	397	384	188	513	112	57	374	512	409	556	281	264	408
4194	66.2	*Selenomonas sputigena*	1	11	22	23	83	16	393	10	1	1	7	4	19	7	15	5
4089	65.2	*Caldithrix abyssi*	170	271	27	24	326	78	372	98	320	116	66	48	240	66	444	104
4236	64.9	*Salmonella enterica*	72	194	10	91	40	112	66	78	190	143	54	57	78	106	135	84
4209	63.7	*Brevibacillus agri*	12	66	48	39	28	38	31	12	63	62	30	36	15	39	24	22
4297	63	*Atopobium parvulum*	22	33	63	69	113	35	196	65	52	37	48	28	54	45	51	34
4155	58.9	*Geobacter bemidjiensis*	37	12	15	41	16	10	33	10	28	11	4	1	3	5	3	1

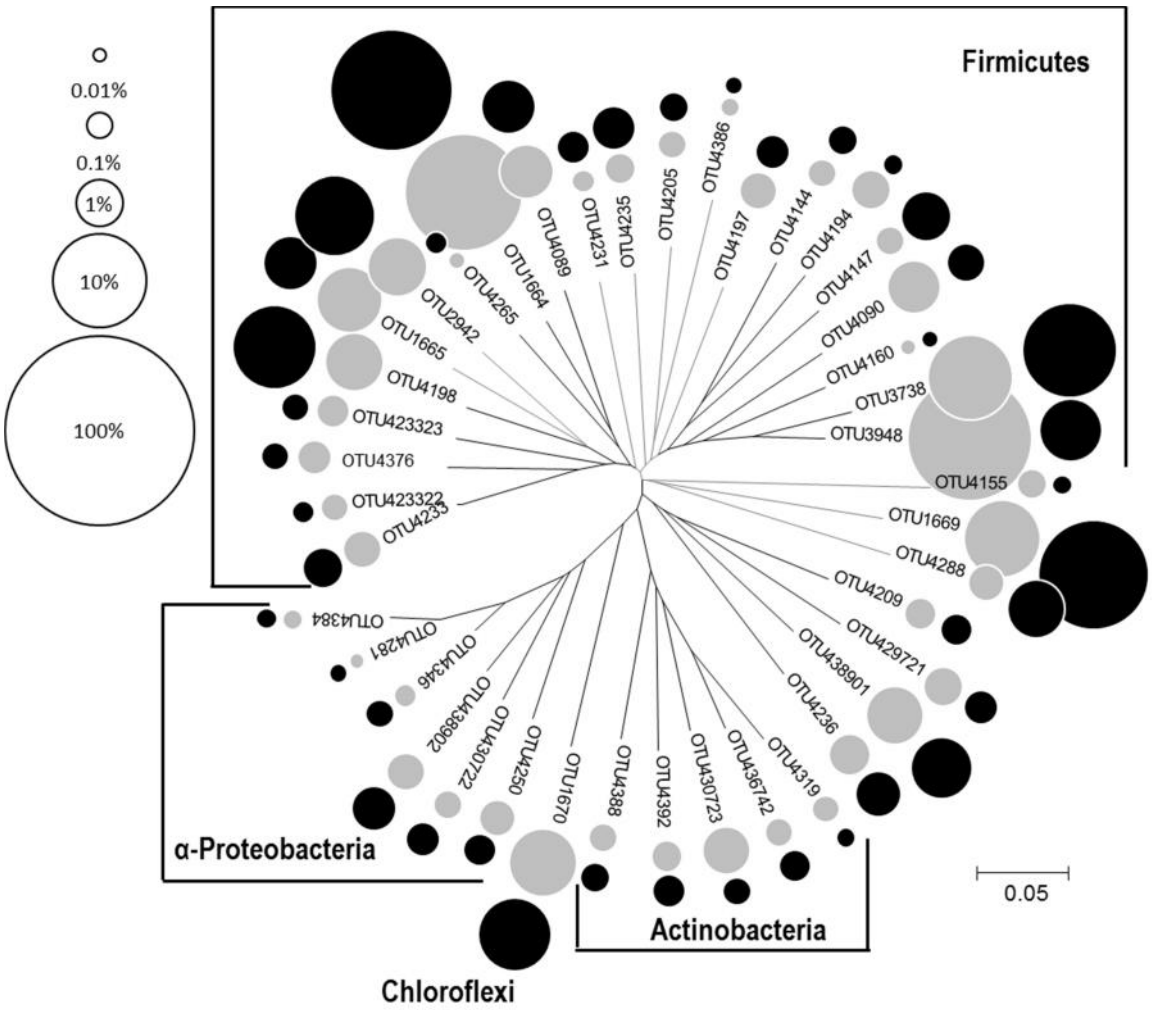

Figure 2-4 Phylogenetic distribution and relative abundances of core OTU in the pooled pyrosequencing libraries for Trial 1 (grey) and Trial 2 (black). OTU sequences were aligned using Clustal in MEGA 4.0 and clustered based on the neighbor-joining method, with bootstrap values calculated based on 500 replicates. Branch segments originating from unsupported nodes (bootstrap value<50%) are indicated in grey. The relative abundance of each OTU within the pooled pyrosequencing libraries for each trial is proportional to the diameter of the circles. These OTU were present in all digesters, and accounted for 92% and 95% of the total pyrosequencing reads in Trial 1 and Trial 2 respectively. Detailed abundance and taxonomic information are available in Table 2-5.

Type II (archaeal thermosome) community analysis

The thermosome clone library sequences assembled into 7 OTU, with OTU501 (most similar to *Methanothermobacter marburgensis*, 89% identity), and OTU795 (most similar to *Methanosarcina barkeri*, 88% identity), being numerically dominant. The remaining 5 OTU were most closely related to *M. marburgensis* (4) and *Methanospirillium* sp. OTU-specific quantitative PCR assays targeting the two most abundant OTU in the clone library indicated that both the hydrogenotrophic and acetoclastic methanogens were present in roughly equal numbers, suggesting that during the digestion period, both the hydrogenotrophic and acetoclastic methanogenic pathways were represented (Figure 2-5). The utilization of both methanogenesis production pathways is likely a characteristic of a more robust digester community, as it can respond to operational changes, input composition or toxin exposure more readily. Previous work characterizing digesters processing ethanol stillage waste and manure only showed evidence of microorganisms associated with hydrogenotrophic methane production, which may provide an explanation for the collapse of methanogenesis in those reactors (Town et al. 2014b).

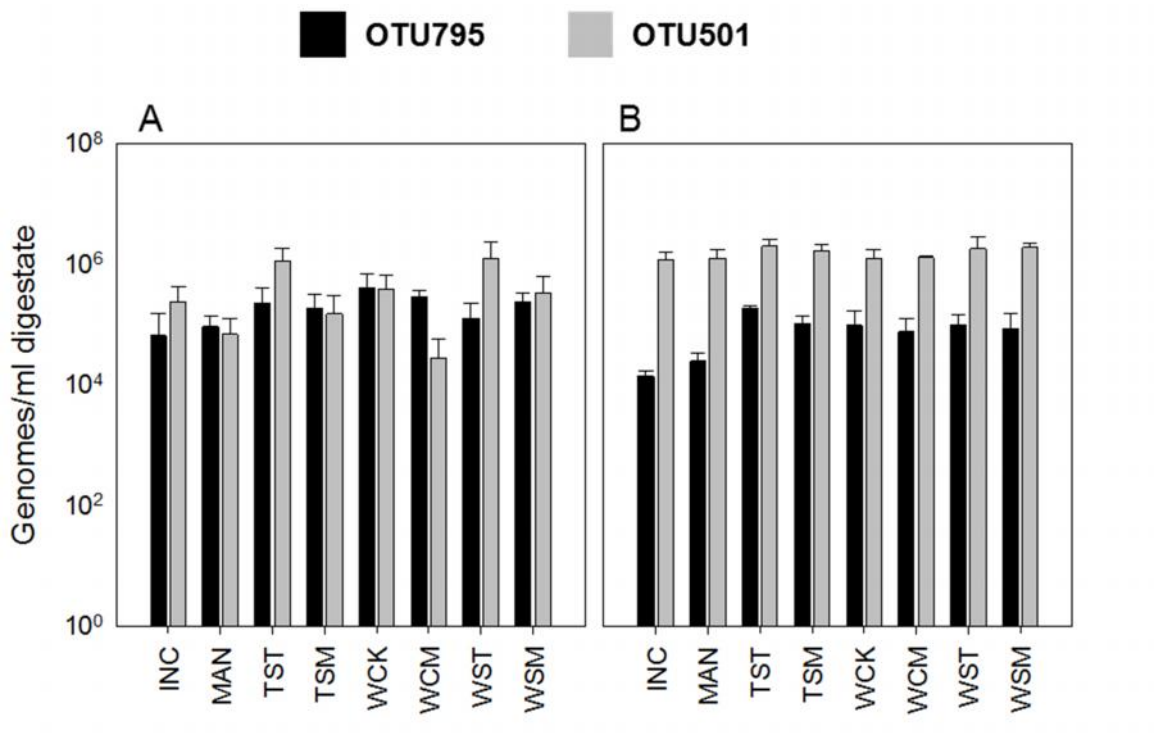

Figure 2-5 Average abundance of OTU795 (Methanosarcina barkeri, 88%) (A, B) and OTU501 (*Methanothermobacter marburgensis*, 89%) (C, D). OTU-specific qPCR assays were used to quantify selected organisms in end-product digestate from triplicate reactors processing inoculum (INC), thin stillage (TST), wetcake (WCK) or whole stillage (WST) either alone (black) or in combination with manure (white) at a 1:1 (A,C) or 2:1 (B,D) ratio of ethanol byproduct:manure.

2.6 Conclusions

Dry-grind wheat ethanol waste streams were suitable for methane production using thermophilic AD. Co-digesting ethanol byproducts with manure resulted in a more consistent and faster rate of methane accumulation. Co-digesting thin stillage and manure produced more methane than expected given the BMP of the individual substrates. Microbial community analysis of the end product digestate revealed a consistent community composition across input combinations. Microorganisms critical to both hydrogenotrophic and acetoclastic methanogenesis pathways were abundant in all reactors. Manure addition increased bacterial diversity, although it is not clear if these changes directly resulted in increased reactor performance.

2.7 Acknowledgments

The authors are grateful to Bonnie Chaban for her help in generating the archaeal thermosome clone libraries. Funding for this research was provided by the Agricultural Biofuels Innovation Project (Agriculture and Agri-Food Canada).

CHAPTER 3 - Molecular characterization of anaerobic digester microbial communities identifies microorganisms that correlate to reactor performance

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Citation

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Authors contributions

Conceived and designed the experiments: JRT TF TJD. Performed the experiments: JRT TJD. Analyzed the data: JRT MGL TJD. Wrote the paper: JRT TJD.

Chapter 3 Preamble

Analysis of the end-point digestate provided an inventory of microorganisms that could play a significant role in methane production in this system. The identification of a numerically dominant core microbiome present in all reactors regardless of input material suggested that the thermophilic conditions exert strong selective pressure on the community, and that there may be some phylogenetic consistency in the microbial communities of reactors operated under similar conditions. In order to confirm the correlation between individual organisms and specific biochemical steps during methanogenesis, a time-course study was designed with digestate sampled at each biochemical stage: hydrolysis, acidogenesis, acetogenesis and methanogenesis.

3.2 Abstract

A time-course analysis was conducted of thermophilic anaerobic digestion of dairy manure and wheat distillery thin stillage. Sequencing of chaperonin targets provided a phylogenetic survey of both Bacteria and Archaea in the digestate, along with an appraisal of the diversity of the reactor microbiome. A total of 1129 bacterial operational taxonomic units (OTU) were detected in the reactors, with OTU related to *Clostridium* becoming numerically dominant by day 7, and *Acetivibrio*-related OTU by day 35. Archaeal communities were less diverse, with 19 OTU detected representing both acetoclastic and hydrogenotrophic methanogens. Regardless of input material, the same organisms came to dominate the reactors, reflecting strong selective pressures present in the digesters. Principal coordinate analysis of the microbial communities showed that the bacterial communities clustered based on factors other than input material. Bacterial and Archaeal OTU were identified with significant correlations to performance parameters, suggesting important roles in the methane production pathway.

3.3 Introduction

If sufficiently optimized, anaerobic digestion (AD) of agricultural waste is an efficient waste disposal system and a source of renewable energy. The process is dependent on the metabolic activity of a complex microbial consortium to convert the input material, which may consist of agricultural, animal or food processing waste, into methane gas. Optimizing the operation conditions to favor the growth and metabolic activity of organisms that break down the organic input material to produce specific end products is desirable; however, a lack of detailed understanding of these microbial communities has hindered progress in this regard (Dar et al. 2008). The current practice of modifying

organic loading rates or altering the pH of the digestate has yielded mixed results (Werner et al. 2011; Westerholm et al. 2012a). Moreover, a lack of tools for directly monitoring the composition of the digester microbiome further complicates the situation with drops in methane production and spikes in volatile fatty acid (VFA) accumulation often going unexplained (Ward et al. 2008).

Although most AD is maintained at mesophilic temperatures, thermophilic conditions provide the most thorough breakdown of the organic inputs. When sufficiently optimized thermophilic AD is more efficient and requires shorter hydraulic retention times; however, the thermophilic microbial community has been shown to be less diverse, more unstable, and more sensitive to fluctuations in operational parameters (Weiland 2010). Grain ethanol distillery waste products, such as those generated from corn, wheat, and barley, can be converted to methane under thermophilic conditions can provide a source of relatively stable and nutrient rich organic material that might otherwise be a waste product (Ziganshin et al. 2011). The distillation process during ethanol production consumes essentially all of the available six carbon sugars in the stillage, converting them to ethanol using yeast fermentation. The remaining stillage waste material contains predominantly five carbon sugars, complex carbohydrates such as cellulose, lipids and proteins (Mustafa et al. 2000). Studies examining the biogas potential of this substrate have shown the process to be energy efficient in terms of carbon balance, especially when the energy produced from the digester is used to offset energy expenditures during ethanol production (Agler et al. 2008; Eskicioglu et al. 2011; Schaefer and Sung 2008). Recently, studies have examined the co-digestion of stillage waste with manure as a way

to boost methane production as well as increase the stability and consistency of the AD process (Westerholm et al. 2012a).

Previous studies examining the composition and dynamics of the bacterial communities associated with thermophilic AD reactors have left many unanswered questions. While microbial communities appear to undergo large shifts in species diversity over the short term, they show surprising robustness and consistency over the long term, even after changes in operating parameters or exposure to toxins (Schauer-Gimenez et al. 2010; Werner et al. 2011). Many of these studies have been unable to show a consistent relationship between microbial composition and digester performance, in particular methane production and volatile solids consumption (Krause et al. 2008; Liu et al. 2009; Wang et al. 2009). More recently, molecular characterization of digester communities combined with quantitative PCR assays have successfully correlated specific microorganisms to digester performance parameters including methane production and volatile fatty acid catabolization (Lv et al. 2013).

During AD, organic material is converted to methane by a microbial consortium consisting of both Bacteria and methanogenic Archaea. Anaerobic bacteria initially degrade the substrate by hydrolysis and acidogenesis. The end products of this initial breakdown are CO_2, H_2 and VFA including acetate, propionate, butyrate, formate, succinate and lactate. Acetogenic bacteria further oxidize the VFA, generating acetate, CO_2 and H_2. The final stage, methanogenesis, is achieved through the metabolic activity of acetoclastic or hydrogenotrophic methanogens. Acetate can be converted to CH_4 directly by acetoclastic methanogens, of which Methanosarcina and Methanosaeta are the most frequently described (Demirel and Scherer 2008). Alternatively acetate can be

oxidized to CO_2 and H_2 by bacteria in a syntrophic association with hydrogenotrophic methanogens (e.g. Methanothermobacter, Methanoculleus) (Demirel and Scherer 2008). To achieve optimal methane production in this system, balance must be maintained between bacterial and archaeal metabolic activity. An increase in metabolic intermediates can be inhibitory to other critical organisms in the consortium, and result in reduced reactor performance or a complete collapse of methanogenesis. A better understanding of the specific microorganisms that are essential at each stage of methanogenesis and their interaction with each other is critical for optimizing reactor design and operation as well as troubleshooting issues with regard to reactor performance.

Molecular methods, including universal target amplification combined with pyrosequencing and quantitative PCR, allow analysis of the microbial community at a resolution that can distinguish between closely related species, and at a depth that permits detailed examination of community structure parameters such as richness and diversity. A protein coding gene, chaperonin 60 (*cpn*60) is universally conserved among eukaryotes, bacteria and some archaea and, while there are exceptions, is more commonly present as a single copy gene, allowing for accurate quantification of organisms (Hill et al. 2004). Type I chaperonins (*cpn*60) are present in bacteria and some archaea, have been shown to provide greater resolution between closely related organisms compared to 16S rRNA-encoding genes, and have recently been proposed as a suitable molecular barcode for Bacteria using the International Barcode of Life criteria (Links et al. 2012). Type II chaperonins, or thermosomes, are present in archaea and the eukaryotic cytosol and universal primers have recently been developed for amplifying

this target from mixed microbial communities (Chaban and Hill 2012) . A database of reference type I and type II chaperonin sequences (www.cpndb.ca) provides a breadth of reference sequences on par with that available for 16S rRNA-encoding sequences (Hill et al. 2004). These tools have been exploited to examine microbial communities from a variety of environments (Chaban and Hill 2012; Dumonceaux et al. 2006), but no previous studies have examined both type I and type II chaperonins in mixed bacterial/archaeal communities such as those associated with AD.

A time-course analysis was performed of bacterial and archaeal communities within thermophilic digesters processing wheat ethanol stillage and dairy cattle manure, and used molecular methods to quantify and monitor organisms critical in the methanogenesis pathway. Ecological parameters of the microbial communities were examined (evenness, richness, and diversity), as these have been shown previously to affect reactor performance, especially as it relates to reactor variability (Schauer-Gimenez et al. 2010; Werner et al. 2011). The information gained by characterizing the microbiome of both high- and low-performing digesters will help to identify a target microbial population and composition associated with maximum reactor performance. The data can also be used to inform reactor design and dictate the operational parameters for introducing and recycling microorganisms during digestion.

3.4 Materials and Methods

Input materials

Wheat grain distillery thin stillage was obtained from Terra Grains Inc. (Moose Jaw, SK, Canada), a facility producing ethanol from dry-ground wheat grain. Manure was

collected from dairy cattle (University of Saskatchewan, Saskatoon, SK, Canada). The starter inoculum (INC) was generated by incubating dairy cattle manure anaerobically at 55°C for two weeks prior to beginning the trial. Total (TS) and volatile (VS) solids for each of the input materials were determined using standard protocols (Association, 1995). Values for %VS and %TS of the input material are listed in Table 3-1.

Table 3-1 The total (TS) and volatile (VS) solids composition of reactor input material.

	%TS	%VS (of TS)
Inoculum (INC)	12.8	80.9
Manure (MAN)	10.9	81.7
Thin Stillage (TST)	10.9	88.3

Bench-scale AD

Bench-scale thermophilic reactors digesting inoculum (INC), dairy manure (MAN), wheat grain distillery thin stillage waste (TST) or thin stillage combined with dairy cattle manure (TSM) were set up in 1 L glass bottles and sealed with air-tight lids fitted with silicone septae. Dairy manure or thin stillage were added individually or mixed in a 1:1 ratio of stillage:manure based on VS content. The stillage or stillage/manure combinations were then mixed with a starter inoculum in a 1:1 ratio based on VS content. The total volume of material in each digester was adjusted to 300 mL and ~5% TS with sterile water. All input combinations were run in duplicate. The initial pH of the digestate mixtures ranged from 7.4-9.3. Bottles were sealed and flushed with N_2 gas at 82.74 kPa (12 psi) for 5 minutes using an outlet needle before being bled down to 3.45 kPa (0.5 psi), and incubated at 55°C for 48 days in an anaerobic chamber with an atmosphere of 85% N_2, 10% H_2 and 5% CO_2.

Sample Collection

Biogas accumulation in the digesters was monitored at 55ºC every 2-3 days using a pressure transducer (Sper Scientific, Scottsdale, AZ,USA) equipped with a 25G needle, and volume measurements were calculated at standard temperature and pressure. G as samples were taken, as needed, when reactor pressure exceeded 34.5 kPa (5 psi). Gas samples were extracted using a 20mL syringe equipped with a stopcock (Cole Parmer, Vernon Hills, IL, USA) and 25G needle and immediately transferred to a 5mL evacuated, dehumidified vial and stored at 4°C until analysis. Following gas sample extraction, digestate samples were collected by removing the reactor lid within an anaerobic

chamber and sampling 3 mL of digestate using a wide-bore pipette. Samples were stored at -80°C until DNA extraction. Reactors were then flushed with N_2 gas at 82.74 kPa (12 psi) for 5 minutes using an outlet needle before being bled down to 3.45 kPa (0.5 psi) and returned to the incubator.

Biogas analysis

The relative percentage of CO_2 in each gas sample was quantified using a Varian Micro-GC (CP-2003, Agilent, Santa Clara, CA, USA) equipped with a 10 m Poraplot U column and thermal conductivity detector (TCD). Relative percentages of O_2, N_2, and CH_4 were quantified using a 10 m molecular sieve column and TCD. Injector and column temperatures were 110°C and 100°C respectively and the equipment was calibrated using certified standards.

Substrate analysis

Digestate samples taken as described above were diluted 1:5 in water, centrifuged, and loaded onto an HPLC (#1515, Waters, Milford, MA, USA) equipped with an Aminex HPX-87H column (Biorad, Hercules, CA, USA). Sample peaks were identified and quantified by comparison to standard curves for formate, succinate, lactate, acetate, propionate, and butyrate.

Universal target amplification and sequencing

Total genomic DNA was extracted from end product digestate samples using a modified bead beating method as described previously (Dumonceaux et al. 2006). The cpn60 (type I chaperonin) and thermosome (type II chaperonin) universal targets were amplified

using multiplex identifier (MID) tagged primer cocktails and amplification conditions previously shown to maximize the range of organisms detected (Chaban and Hill 2012; Hill et al. 2005). All primer sequences are listed in Table 3-2. For amplification of the type I cpn60 target, each reaction contained: 1X PCR buffer (Invitrogen, Carlsbad, CA, USA), 2.5 mM MgCl2, 0.2 mM each dNTP, 100 nM each of H279/H280, 300 nM each of H1612/H1613 and 1U of Platinum Taq (Invitrogen, Carlsbad, CA, USA) and cycling conditions of 1 x95°C, 5 min; 40x 95°C 30sec, 42-60°C 30sec, 72°C 30sec; 1x 72° 2min. Type II chaperonins were amplified under the same conditions using 437.5 nM each of JH0175/JH178 and 62.5 nM each of JH0268/JH0269 and cycling conditions of 1 x95°C, 5 min; 40x 95°C 30sec, 55°C 30sec, 72°C 30sec; 1x 72° 2min. Amplification products from replicates for each digester condition were pooled and then sequenced using the Roche 454 GS FLX with Titanium chemistry (Branford, CT, USA).

Table 3-2 Primer sequences and amplification conditions for universal target amplification.

Primer Name	Primer Sequence (5'-3')	Amplification conditions
H279	GAIIIIGCIGGIGAYGGIACIACIAC	95°C 5 min (1x) 95°C 30 sec ; 42-60°C 30sec; 72°C 30 sec (40x) 72°C 2 min (1x)
H280	YKIYKITCICCRAAICCIGGIGCYTT	
H1612	GAIIIIGCIGGYGACGGYACSACSAC	
H1613	CGRCGRTCRCCGAAGCCSGGIGCCTT	
JH0175	GGICCIMRRGGIITIGAYAARATG	95°C 5 min (1x) 95°C 30 sec; 55°C 30sec; 72°C 30 sec (40x) 72°C 2 min (1x)
JH0178	GCIAIITCRTCIATICCYTTYTG	
JH0268	GGCCCGAAGGGCATGGACAAGATG	
JH0269	GGCATGTCGTCGATGCCCTTCTG	

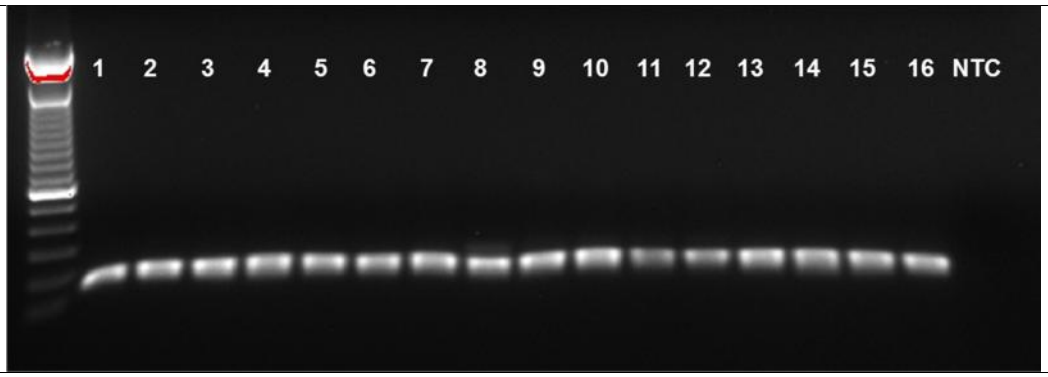
Pyrosequencing data analysis

Sequencing reads were processed using mPUMA (Links et al. 2013) with assembly being performed using Trinity with an Inchworm kmer size of 31bp. OTU-frequency data was calculated using a data set of pyrosequencing reads randomly subsampled to the smallest library size; 8500 for the type I libraries and 3500 for the type II libraries. OTU frequency data was used as input for Mothur, Unifrac, and R to correlate OTU abundance, taxonomic composition and microbial community structure with reactor performance data.

Quantitative PCR assays

OTU-specific primers were designed using Beacon Designer v 7.0 (Premier Biosoft, Palo Alto, CA, USA) such that primer similarity was restricted to the desired OTU, avoiding cross-amplification of other assembled OTU sequences. Primers were validated by amplifying the target from an AD template, cloning the amplicon into pGEM-T Easy vector (Promega, Madison, WI, USA), and sequencing representative clones to verify that only the intended target was amplified. Previously described 16S rRNA-based universal primers for amplifying Bacteria (Lee et al. 1996) were used to estimate total bacterial load in the digestate. Novel primers for estimating total archaeal load were designed based on all publically available aligned 16S sequences. Universal archaeal primers were validated by amplifying genomic DNA from a taxonomically diverse range of Archaea (Figure 3-1). Additionally, amplicon generated from digestate with an abundance of both Bacteria and Archaea was cloned; 85 clones were sequenced, of which

100% were of archaeal origin (Table 3-3), indicating that the primers were sufficiently specific to avoid bacterial cross-amplification.



Lane	Organism	Phylum	Class
1	*Halobacterium halobium* 34008	Euryachaeota	Halobacteria
2	*Halobacterium salinarum* ATCC 33170D	Euryachaeota	Halobacteria
3	*Halobacterium salinarum* ATCC 33171D	Euryachaeota	Halobacteria
4	*Haloferax volcanii* WR241	Euryachaeota	Halobacteria
5	*Haloferax volcanii* WR536	Euryachaeota	Halobacteria
6	*Methanospirillum hungatei*	Euryachaeota	Methanomicrobia
7	*Methanotorris igneus*	Euryachaeota	Methanococci
8	*Methanococcus maripaludis*	Euryachaeota	Methanococci
9	*Methanococcus vannielii*	Euryachaeota	Methanococci
10	*Methanococcus voltae*	Euryachaeota	Methanococci
11	*Sulfolobus solfataricus*	Crenarchaeota	Thermoprotei
12	*Sulfolobus* sp.	Crenarchaeota	Thermoprotei
13	*Thermoplasma acidophilum*	Euryachaeota	Thermoplasmata
14	*Thermococcus gorgonarius* ATCC 700654D	Euryachaeota	Thermococci
15	*Thermococcus pacificus* ATCC 700653D	Euryachaeota	Thermococci
16	*Thermococcus zilligii* ATCC 700529D	Euryachaeota	Thermococci
17	No Template Control		

Figure 3-1 Amplification of archaeal genomic DNA using universal 16S primers for estimating total archaeal load.

Table 3-3 Clone sequences from universal archaeal primer amplification from a mixed microbial community.

# Reads	Nearest Neighbor	% Identity
19	*Methanosarcina acetovorans*	97%
66	*Methanoculleus marisnigri*	97%

For each qPCR assay, cloned amplicon was used to generate standards from 107 to 101 gene copies per reaction. Target OTU sequences were amplified using EvaGreen qPCR amplification mix (BioRad, Hercules, CA, USA) with 400 nM of each primer; primer sequences and amplification conditions are listed in Table 3-4. Specific OTU, total Archaea, and total Bacteria abundance estimates were correlated to methane proportion in biogas samples, daily methane production, and acetate accumulation by Spearman's Rank analysis using R ($\alpha<0.01$).

Table 3-4 OTU-specific qPCR primer sequences and amplification conditions.

Primer Name	Primer Sequence (5'-3')	Amplification conditions	Target
SRV3-1	CGGYCCAGACTCCTACGGG	95°C 5 min (1x) 95°C 15 sec ; 62°C 20sec ; 72°C 20 sec (40x) 72°C 2 min (1x)	Total Bacteria
SRV3-2	TTACCGCGGCTGCTGGCAC		
D0234	CTTAAAGGAATTGGCGGGG	95°C 5 min (1x) 95°C 15 sec ; 55°C 20sec ; 72°C 20 sec (40x) 72°C 2 min (1x)	Total Archaea
D0235	GGCCATGCACCSCCTCTC		
D0299	CCTAAGCGTTCCCATAGAA	95°C 5 min (1x) 95°C 15 sec ; 55°C 20sec ; 72°C 20 sec (40x) 72°C 2 min (1x)	OTU795
D0300	TTGCCTCTTCCTGGTCTA		
D0301	AGATCGCCATCGACATCA	95°C 5 min (1x) 95°C 15 sec ; 57°C 20sec ; 72°C 20 sec (40x) 72°C 2 min (1x)	OTU805
D0302	CTCGGTGAGCTTCTCCTT		
D0307	CAATGCAGAAGGTTACTT	95°C 5 min (1x) 95°C 15 sec ; 52°C 20sec ; 72°C 20 sec (40x) 72°C 2 min (1x)	OTU2847
D0308	CGGTAAGATTTCCTGAATG		
D0290	CTATACTTCTTGCTCAGG	95°C 5 min (1x) 95°C 15 sec ; 57°C 20sec ; 72°C 20 sec (40x) 72°C 2 min (1x)	OTU2811
D0291	CGATAACATCATCATTGG		
D0309	GAGTTTGTCGCCACCATA	95°C 5 min (1x) 95°C 15 sec ; 53°C 20sec ; 72°C 20 sec (40x) 72°C 2 min (1x)	OTU2898
D0310	AAGCAAATCCTGGACATT		
D0311	GTGAAAGACCTCCTTCCC	95°C 5 min (1x) 95°C 15 sec ; 58°C 20sec ; 72°C 20 sec (40x) 72°C 2 min (1x)	OTU2941
D0312	AACGGCAACCACATTTAG		
D0228	CCGATACCGATAAGATGGA	95°C 5 min (1x) 95°C 15 sec ; 58°C 20sec ; 72°C 20 sec (40x) 72°C 2 min (1x)	OTU2686
D0229	GCGATTATGAGCAGCTTC		

3.5 Results & Discussion

Biogas composition

Biogas composition analysis in digesters processing inoculum (INC) and manure (MAN) showed that the proportion of methane in the biogas peaked at 88% and 83% respectively (Figure 3-2A). Although the methane proportion in digesters processing thin stillage alone (TST) did reach >80% (Figure 3-2A), the daily biogas production trailed those of other inputs (Figure 3-2B). The high proportion of methane being produced in these reactors indicates highly active methanogen populations are present, however the decrease in daily production in comparison to INC and MAN reactors suggests the breakdown of the input material is likely a rate limiting step when processing stillage waste. By contrast, the digesters processing thin stillage with manure (TSM) reached a maximum of ~50% methane in the reactor headspace, declining to 0% methane by day 31. This suggests poor metabolic activity by methanogenic archaea as a rate limiting factor for methane production in these reactors.

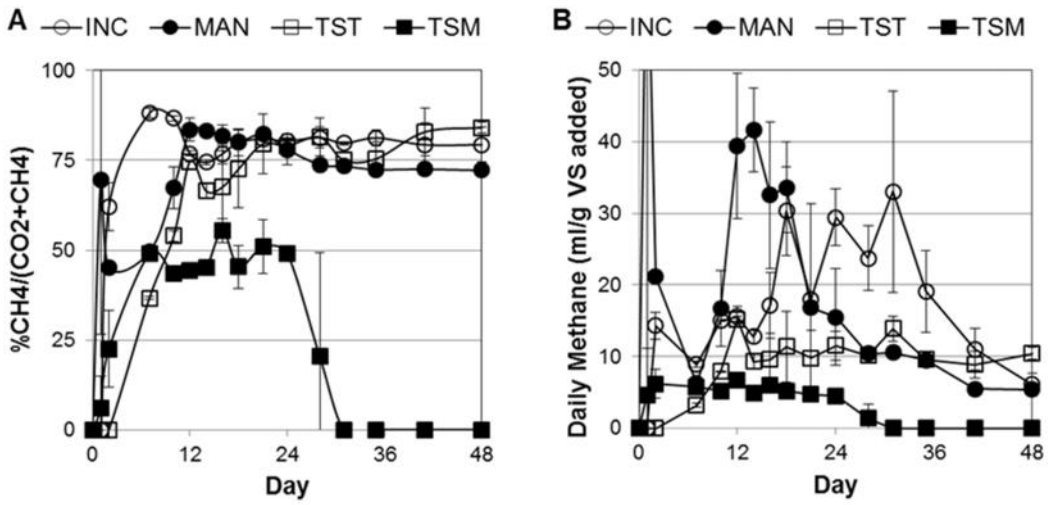


Figure 3-2 Average methane proportion (A) and daily production (B) in reactors processing inoculum (INC), manure (MAN), thin stillage (TST) and thin stillage with manure (TSM). Biogas samples were collected from duplicate reactors, transferred to a dehumidified, evacuated vial, and gas composition was determined using gas chromatography.

The disparity in performance between the TST and TSM digesters provided an opportunity to examine in more detail the environmental and/or microbiological reasons for the failure of the methanogenesis pathway, as the co-digestion of whole stillage from cereals with manure has previously been shown to have good methane production under similar conditions (Westerholm et al. 2012a).

VFA metabolism

Acetate was the most abundant VFA in the reactors, with butyrate and propionate produced in lesser quantities (Figure 3-3). Digestate pH correlated negatively with VFA accumulation, particularly with acetate accumulation (Pearson correlation = -0.816, p-value<0.01). Formate, succinate and lactate accumulation was not detectable in all digesters. The MAN reactors were the most efficient at catabolizing VFAs, completely eliminating VFA accumulation in the digestate after day 21. While the early accumulation of VFA was similar for the INC and TST reactors, the TST reactors were much less efficient at converting VFA to methane, resulting in continued VFA accumulation at the end of the trial period. The TSM reactors showed almost no VFA catabolization, resulting in very poor methane production. These data suggest that in the TSM reactors, the initial steps of the methanogenesis pathway (hydrolysis, acidogenesis, and acetogenesis) were successful and the root cause of the performance disparity was the transition from acetogenesis to methanogenesis, which would be presumed to be caused by a dysbiosis in those reactors.

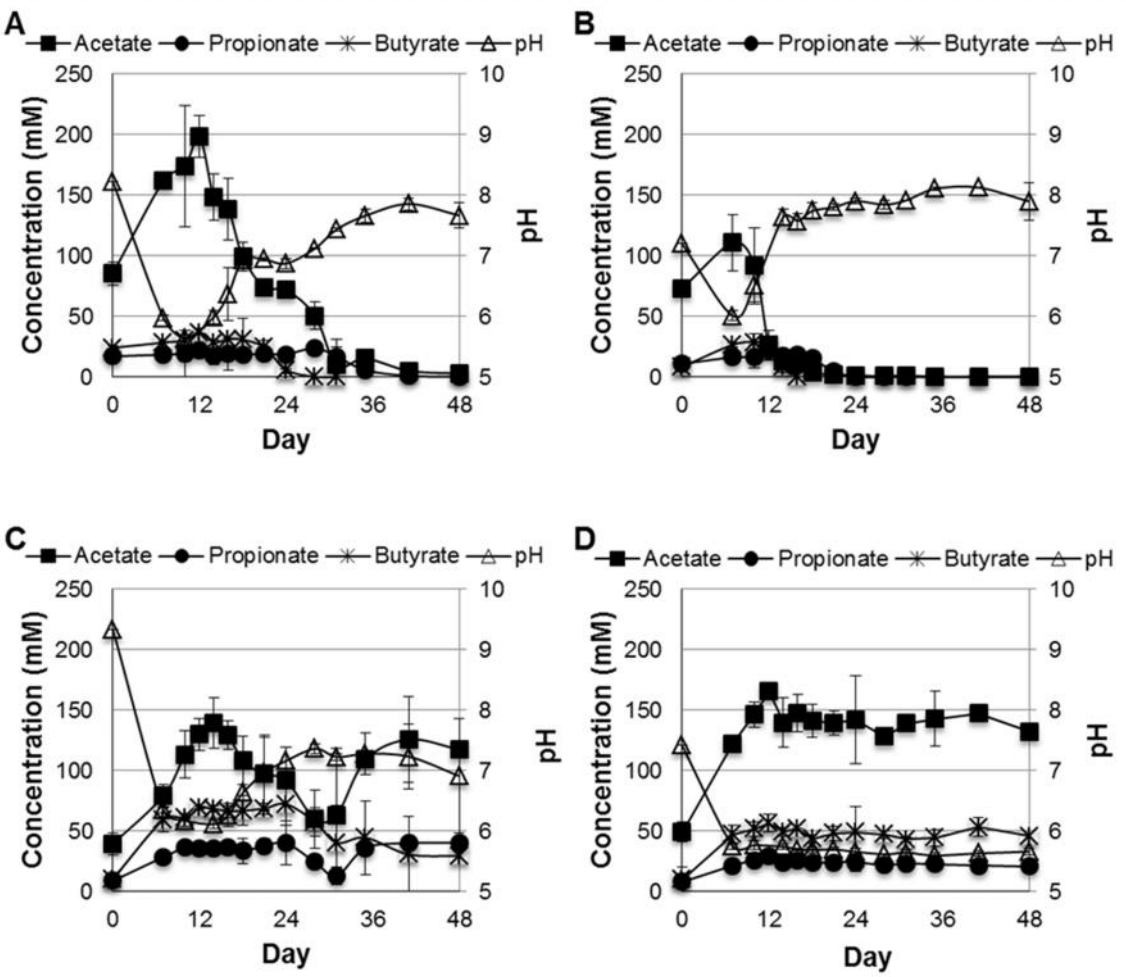


Figure 3-3 Average VFA accumulation in digesters processing INC (A), MAN (B), TST (C) and TSM (D). Digestate samples were collected from duplicate reactors, diluted, filtered, and volatile fatty acid concentration was measured using HPLC.

Microbial community diversity

The addition of manure, a rich source of microorganisms, to the input mixture increased the initial bacterial community richness and evenness as measured by Chao1 and Simpson's indices for the type I (cpn60) target (Figure 3-4). After the first week of incubation, the selective pressure from both the thermophilic environment and the composition of the input material decreased the richness dramatically, particularly in reactors processing thin stillage.

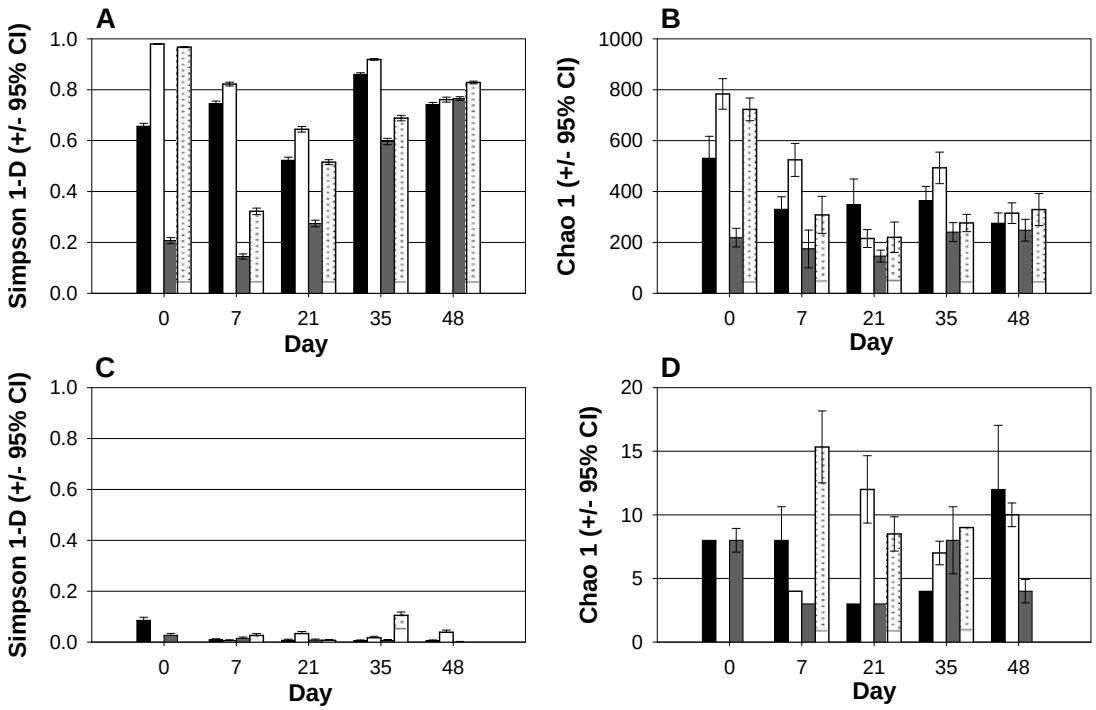


Figure 3-4 Alpha diversity measures for the bacterial (A, B) and archaeal digestate communities (C, D). Subsampled OTU-frequency data was used as input for Mothur to measure community richness (Chao1) and evenness (Simpson's Index) in digesters processing INC (■), MAN (□), TST (▩), and TSM (▨) .

Rank-abundance curves showed that the minimum bacterial diversity was reached at day 7 for TST and TSM reactors and day 21 for INC and MAN reactors (Figure 3-5). By day 48, all digester inputs showed an increase in diversity resulting from both a decrease in abundance of the most dominant OTU, as well as an increase in the number of different OTU detected in the pyrosequencing libraries. These dynamic changes in community diversity are likely the result of proliferation of organisms that are adapted to the selective pressures in this environment including thermophilic temperatures, high concentrations of volatile fatty acids, and low pH. This pattern of selective microorganism proliferation during anaerobic digestion has been seen previously, with decreasing richness and increasing evenness observed over the course of thermophilic digestion (Gannoun et al. 2013).

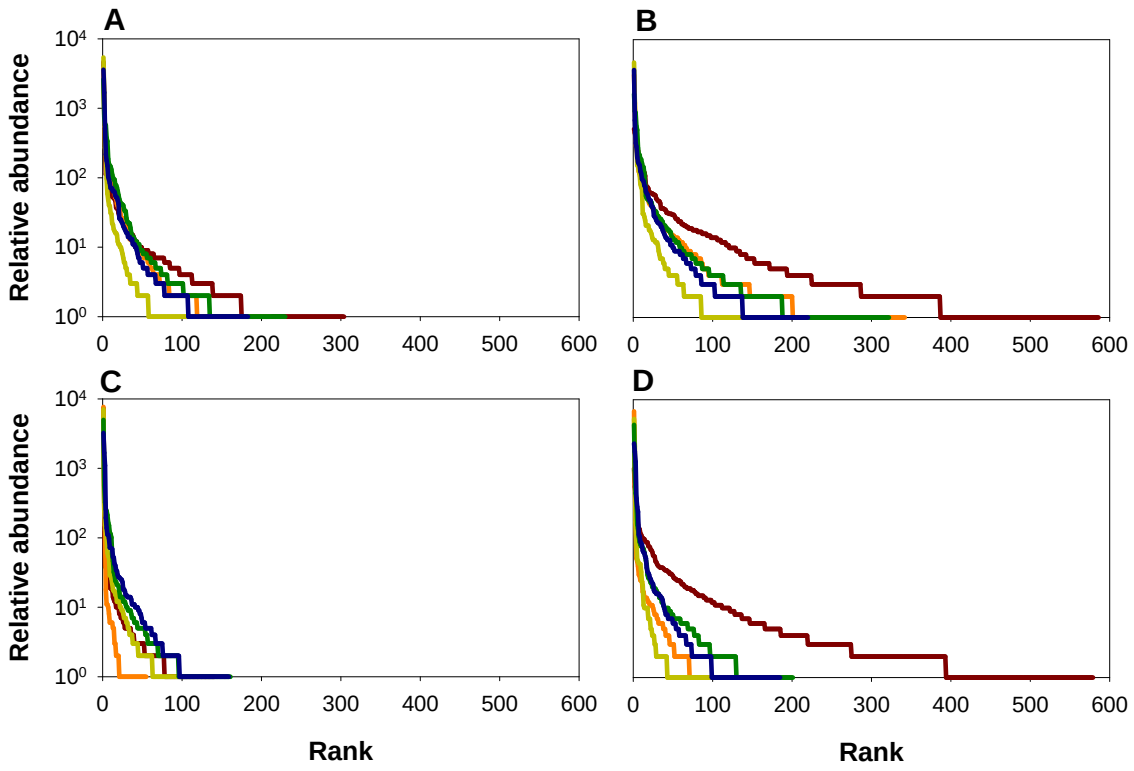


Figure 3-5 Rank-abundance curves for OTU frequency in the pyrosequencing libraries from INC (A), MAN (B), TST (C) and TSM (D) digesters, for days 0 (red), 7 (orange), 21 (yellow), 35 (green) and 48 (blue). OTU-frequency data from subsampled pyrosequencing libraries was used to calculate rank-abundance using Mothur.

The archaeal communities were much less diverse and showed very little change in diversity over the course of the trial as measured by Chao1 and Simpson's indices for the type II chaperonin target (Figure 3-6).

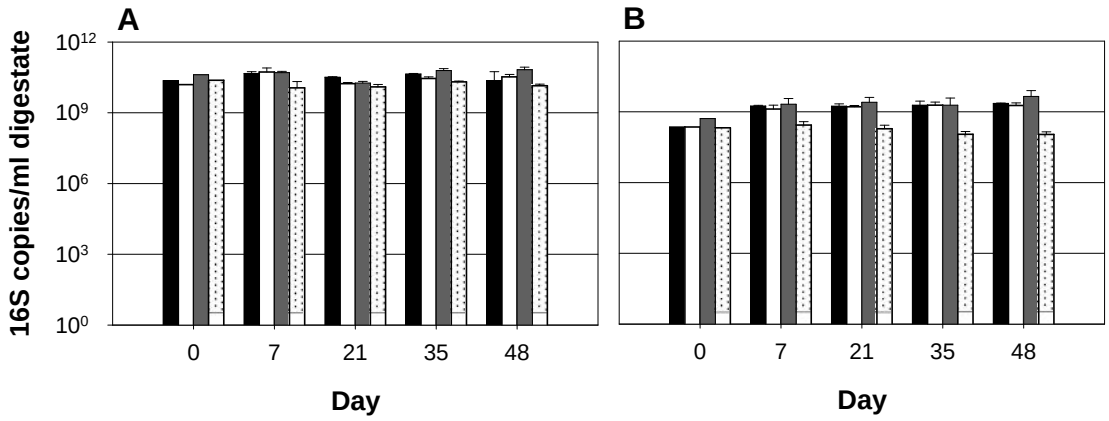


Figure 3-6 Average bacterial (A) and archaeal load (B) was estimated using universal quantitative PCR assays in duplicate reactors processing INC (■), MAN (□), TST (▩), and TSM (▨).

Microbial community composition

Bacterial (type I chaperonin) and archaeal (type II chaperonin) pyrosequencing data was assembled into 1129 and 19 OTU respectively. The relative frequencies of all assembled type I cpn60 OTU in each digester are shown in Figure 3-7. By day 7, the bacterial pyrosequencing libraries were dominated by a single OTU, comprising 39-93% of the total reads regardless of the initial inputs. This OTU, most closely related to *Clostridium leptum* (74% identity), was most abundant in the TST digesters, and is likely critical to the breakdown of the organic material. Other *Clostridium* spp. have been previously associated with thermophilic digestion of stillage waste from rapeseed fermentation (Luo et al., 2011), and several strains isolated from digester sludge processing other waste streams have shown cellulolytic properties (Johnson et al. 1981; Madden 1983; Sleat et al. 1984), producing significant amounts of hydrogen (Levin et al. 2006). Their abundance in this digester system is consistent with high levels of VFA accumulation and their hypothesized role as contributors to hydrogenotrophic methane production.

Pyrosequencing libraries from the INC digesters remained dominated by *Clostridium* spp. throughout the trial while libraries from the MAN, TST and TSM digesters shifted to a community profile dominated by a single OTU most closely related to *Acetivibrio celluylolyticus* (78% identity) by day 35 (Figure 3-7).

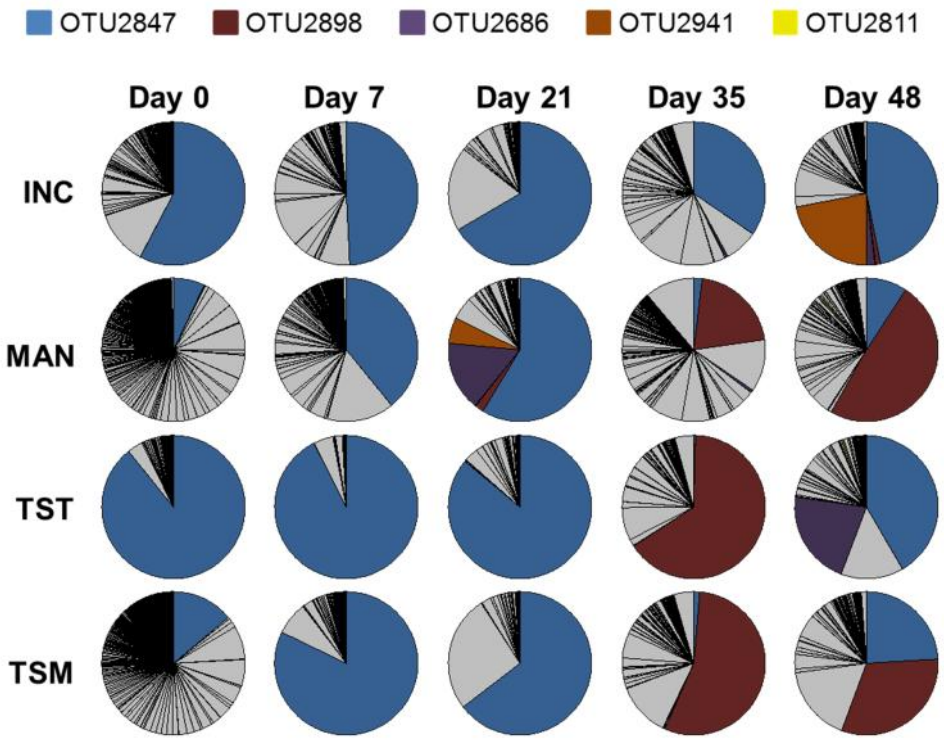


Figure 3-7 Relative OTU distribution in the type I chaperonin (*cpn*60) pyrosequencing libraries. Pyrosequencing reads generated using pooled amplicon from duplicate digesters was assembled using mPUMA, and the relative abundance of individual OTU is shown. OTU sequences were compared to the cpnDB reference database for taxonomic identification: OTU2847 (75% *Clostridium leptum*), OTU2898 (78% *Acetivibrio cellulolyticus*), OTU2686 (75% *Clostridium thermocellum*), OTU2941 (74% *Heliobacterium modesticaldum*), and OTU2811 (85% *Clostridium thermocellum*). OTU frequency data to identify OTU with very low abundance is available in Table 3-5.

Table 3-5 OTU frequency data from type I (*cpn*60) pyrosequencing libraries.

		OTU2847	**OTU2898**	**OTU2686**	**OTU2941**	**OTU2811**
INC	Day 0	4643	3	0	0	0
	Day 7	3841	0	4	0	0
	Day 21	5375	0	0	0	0
	Day 35	2611	2	42	0	23
	Day 48	3591	84	149	1682	18
MAN	Day 0	516	1	2	0	0
	Day 7	3104	2	0	0	18
	Day 21	4662	156	1243	469	13
	Day 35	149	1638	50	0	17
	Day 48	687	3653	20	0	38
TST	Day 0	7357	0	0	0	0
	Day 7	7680	0	0	0	0
	Day 21	7040	0	0	0	0
	Day 35	29	4989	3	0	4
	Day 48	3237	2	1671	13	40
TSM	Day 0	1020	2	1	0	0
	Day 7	6783	0	0	0	0
	Day 21	5279	0	0	0	0
	Day 35	106	4348	23	0	20
	Day 48	1775	2334	1	0	5

The archaeal communities in these digesters showed very little diversity, and the type II chaperonin libraries were dominated by a single OTU that most closely resembled the hydrogenotrophic methanogen *Methanoculleus bourgensis* (90% identity). The second-most prevalent OTU was most closely related to an acetoclastic methanogen, *Methanosarcina barkeri* (88% identity). Together, these two OTU accounted for 95.6-99.9% of the total pyrosequencing reads in the type II chaperonin library (Figure 3-8).

Regardless of the composition of the input material or the initial microbial community distribution, the same organisms came to dominate the reactors, reflecting the strong selective pressures present in the thermophilic digestion environment, a phenomenon previously observed in digesters processing agricultural wastes (Ziganshin et al. 2013).

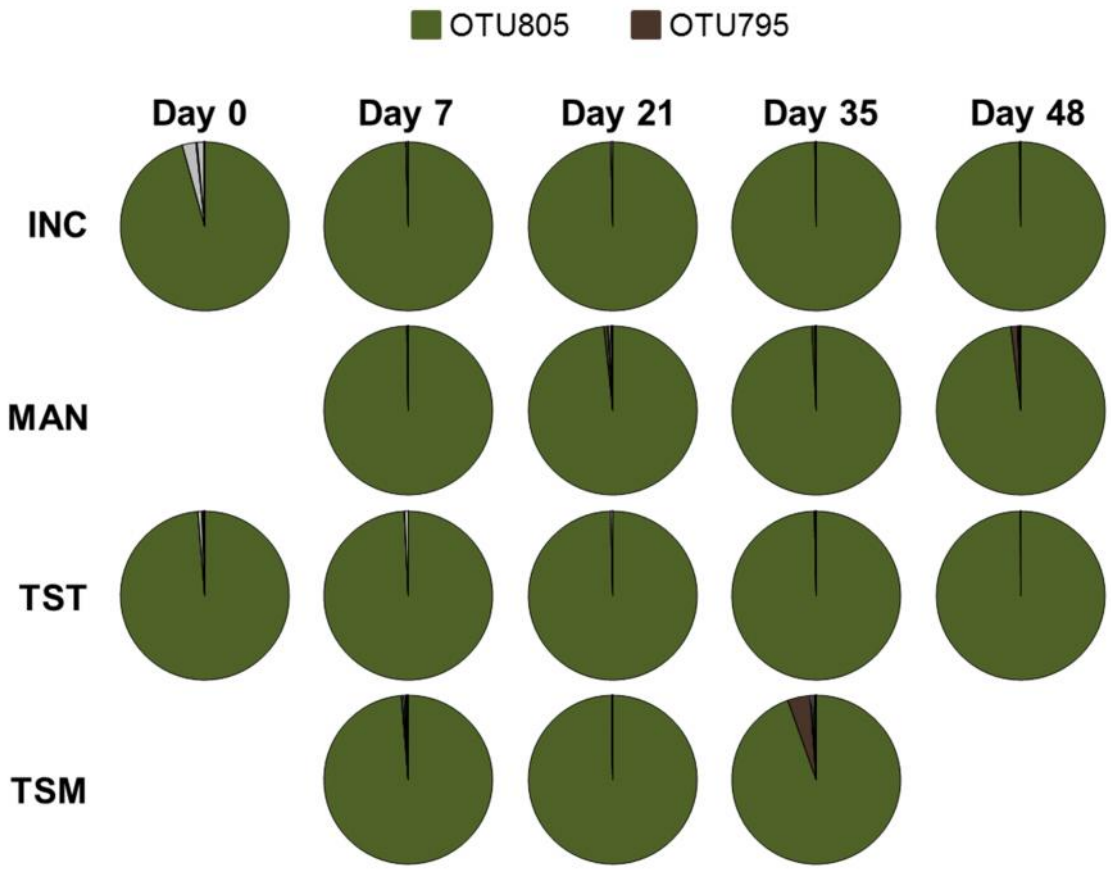
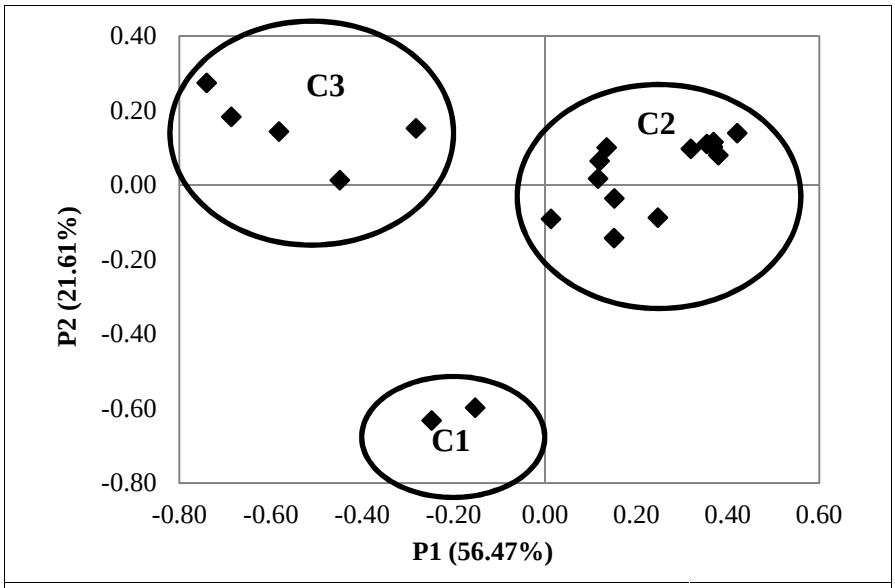


Figure 3-8 Relative OTU distribution in the type II chaperonin (thermosome) pyrosequencing libraries. Pyrosequencing reads generated using pooled amplicon from duplicate digesters was assembled using mPUMA, and the relative abundance of individual OTU is shown. OTU sequences were compared to the cpnDB reference database for taxonomic identification: OTU805 (90% *Methanoculleus bourgensis*), and OTU795 (88% *Methanosarcina barkeri*).

Unifrac

Principal coordinate analysis (PcoA) of weighted Unifrac values of the type I pyrosequencing data confirmed that bacterial communities clustered based on their point in the methanogenesis pathway as opposed to input material, with 78% of the variability explained by the first two coordinates. Samples from days 7 and 21 were more similar to each other than to days 35 and 48, regardless of input material (Figure 3-9). Over the course of the trial, the inoculum-only digesters showed a more consistent community composition compared to the other inputs. Digesters processing either thin stillage, manure or both showed a dramatic shift in bacterial community composition at Day 35. This clustering may indicate that environmental conditions such as temperature, pH and VFA concentration have a stronger selective effect on microbial community composition than input material and that these dynamic community shifts may be relatively consistent across reactors containing different types of stillage and other cellulosic wastes. Recognizing that a single community composition could be highly effective for processing a variety of inputs, could inform the design of a more robust and universal reactor for anaerobically digesting agricultural wastes.



Treatment	Cluster1	Cluster2	Cluster3
INC		Day 0, 7, 21, 35, 48	
MAN	Day 0	Day 7, 21	Day 35, 48
TST		Day 0, 7, 21, 48	Day 35
TSM	Day 0	Day 7, 21	Day 35, 48

Figure 3-9 Weighted Unifrac analysis of digestate bacterial communities. Subsampled OTU-frequency and phylogenetic data was used as input for principal coordinate analysis (PcoA) of weighted Unifrac values.

Correlation of OTU abundances to reactor performance parameters

The pyrosequencing libraries were used as a starting point for identifying organisms critical to methanogenesis in these reactors. OTU frequency was correlated to reactor performance data using Spearman's Rank correlation ($\alpha<0.05$), and OTU with significant correlations to performance objectives were subjected to further analysis. OTU2847 and OTU2898 were also selected for further analysis based on their numerical dominance. While these two OTU did not correlate to performance parameters, pyrosequencing analysis uses end-point PCR amplicon, and its limited dynamic range for detecting differences in abundance is exacerbated when organisms are highly abundant. Selected organisms, along with total Archaea and Bacteria, were quantified in reactor replicates using OTU-specific qPCR assays (Figure 3-10), and their abundances were correlated to methane and acetate accumulation at critical time points using Spearman's Rank correlation (Table 3-6).

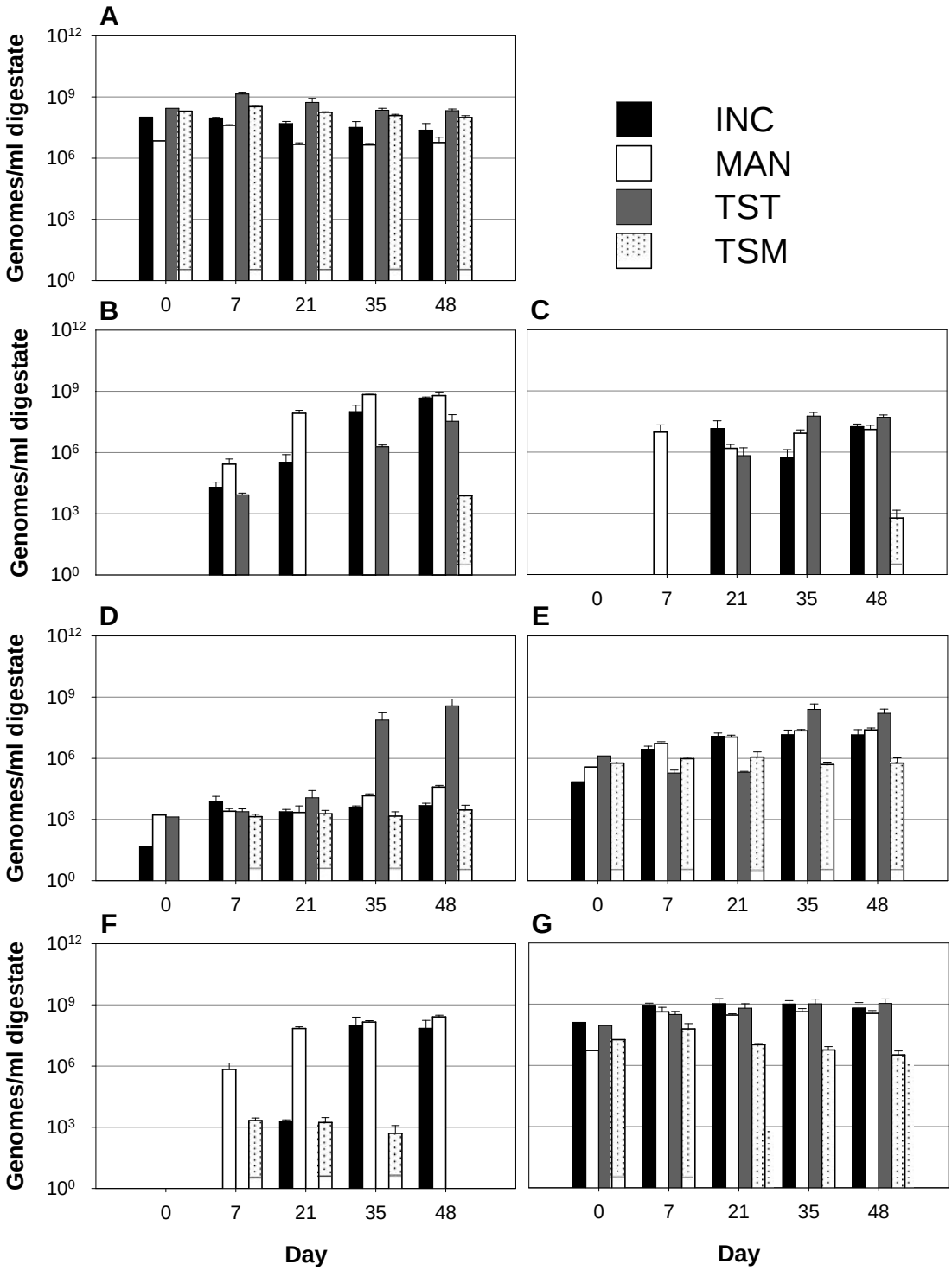


Figure 3-10 Average abundance of selected OTU in reactors processing INC, MAN, TST and TSM. OTU-specific quantitative PCR assays were used to quantify selected organisms in digestate from duplicate reactors at five time points during digestion for OTU2847(A), OTU2898(B), OTU2811(C), OTU2941(D), OTU2686I, OTU795(F), and OTU805(G).digestion.

Table 3-6 Spearman's Rank analysis correlating OTU abundance and reactor performance data. OTU abundance, along with total Archaea and total Bacteria, was estimated in biological replicates using specific quantitative PCR assays. Results were correlated to daily methane production (ml/g VS added), methane proportion of biogas (% $CH_4/(CH_4+CO_2)$), and acetate concentration using Spearman's Rank (ρ) analysis using R (p-value<0.01).

		vs. Daily Methane	**vs. % Methane**	**vs. [Acetate]**
	Nearest Neighbor (%ID)	**ρ (p-value)**	**ρ (p-value)**	**ρ (p-value)**
OTU2847	*Clostridium leptum* (74%)	-0.248 (0.141)	-0.261 (0.197)	0.524 (0.002)
OTU2811	*Clostridium thermocellum* (85%)	0.551 (0.001)	0.504 (0.003)	-0.299 (0.076)
OTU2686	*Clostridium thermocellum* (75%)	0.599 (0.000)	0.561 (0.000)	-0.427 (0.011)
OTU2941	*Heliobacterium modesticaldum* (74%)	0.490 (0.004)	0.547 (0.001)	-0.236 (0.161)
OTU2898	*Acetivibrio cellulolyticus* (78%)	0.556 (0.001)	0.550 (0.001)	-0.608 (0.000)
OTU795	*Methanosarcina barkeri* (88%)	0.316 (0.061)	0.242 (0.150)	-0.453 (0.007)
OTU805	*Methanoculleus bourgensis* (90%)	0.749 (0.000)	0.812 (0.000)	-0.257 (0.129)
Total Bacteria		0.340 (0.043)	0.350 (0.038)	-0.066 (0.689)
Total Archaea		0.627 (0.000)	0.698 (0.000)	-0.473 (0.005)

Bacterial OTU (type I chaperonin)

Total bacterial load, as estimated by qPCR (Figure 3-6), did not correlate significantly to any performance parameter (Table 3-6), consistent with what has been seen in other studies (Krause et al. 2008; Liu et al. 2009; Wang et al. 2009). This does indicate however that the proliferation of selected organisms within the bacterial community show a strong positive correlation to reactor performance measures including methane production and acetate production and catabolization.

OTU2847, most closely related to *Clostridium leptum* (74% identity) and the most abundant OTU in the pyrosequencing libraries, was initially present at ~10^7-10^8 genomes/ml digestate, increased 10x after the first week, and then declined to its initial level by day 48. While universally abundant, this OTU did not correlate significantly to methane production, but did have a significant positive correlation to acetate accumulation, and is likely involved in the hydrolytic or acidogenic stages of methanogenesis. Two other *Clostridium*-like OTU, 2811 and 2686 (85% and 75% identity to *Clostridium thermocellum* respectively), may have played a more significant role in maximizing digester performance. OTU2686 was present at ~10^5 genomes/ml digestate at the onset of the trial and continued to increase 100-1000x in the methane-producing INC, MAN and TST reactors while remaining level in the poorly performing TSM reactors. Both showed a strong, positive correlation to daily methane production as well as the proportion of methane in the biogas samples. In addition, OTU2686 correlated negatively to acetate levels. While OTU2686 was present in the TSM reactors, the lack of proliferation of this organism compared to INC, MAN and TST reactors may be indicative of reduced metabolic activity, contributing to the poor performance. This

data suggests a critical role for these *Clostridium*-like organisms in the hydrogenotrophic methanogenesis pathway in this system, potentially as acetate oxidizing syntrophic bacteria, with OTU2811 in particular conspicuously absent in poorly performing digesters.

OTU2898, most closely related to *Acetivibrio cellulolyticus* (78% identity), was not detectable at the onset of the trial, but increased to ~104 genomes/ml digestate by day 7, and continued to increase to ~10^8 genomes/ml digestate by day 48 (Figure 3-10). This OTU showed a significant positive correlation to methane production in this system as well as a strong negative correlation to acetate accumulation, and was also present at much lower levels in the TSM reactors, appearing only at the last time-point in the trial. The qPCR data for this OTU indicates that this organism is more abundant in the INC and TST libraries than the pyrosequencing data would suggest. It is possible that these sequences are under-represented during universal target amplification for pyrosequencing, or that the OTU-specific primers are co-amplifying an additional unknown sequence, despite the precautions taken to design and validate this OTU-specific assay.

OTU2941, most closely related to *Heliobacterium modesticaldum* (74% identity), was present at very low levels at the onset of the trial (~10^2 genomes/ml digestate), and increased ~10,000x in the TST reactors compared to only ~100x in the INC, MAN and TSM reactors (Figure 3-10). While this OTU did have a statistically significant correlation to daily methane production, it was not as strongly positive as those for OTU2686 and OTU2811 (Table 3-6). However, its proliferation in the TST reactors,

particularly in light of the increased performance in comparison to the TSM reactors, suggests a role for this organism in the successful digestion of thin stillage.

Archaeal OTU (type II chaperonin)

In contrast to total bacterial load, total archaeal load as measured by qPCR correlated both positively to daily methane production and negatively to acetate accumulation (Table 3-6). OTU805, corresponding to the dominant hydrogenotrophic methanogen in this system (90% identity to *Methanoculleus bourgensis*) was universally present at 10^7-10^9 genomes/ml digestate, and increased at least 10x in INC, MAN and TST reactors during the trial while decreasing 10x in the TSM reactors (Figure 3-10). The abundance of this OTU had a strong, significant positive correlation to both daily methane production and the proportion of methane in the biogas, but did not correlate to acetate levels in the digestate, suggesting a metabolic role in hydrogenotrophic methanogenesis.

In contrast, OTU795 was only abundant in the most highly productive reactors, INC and MAN, was present at very low levels in the TSM reactors, and not at all in the TST reactors. This OTU did not correlate to daily methane production, but did correlate negatively to acetate accumulation. This suggests that OTU795 (88% identity to *Methanosarcina barkeri*) represents an acetoclastic methanogen, and its proliferation in the INC and MAN reactors likely explains the complete consumption of acetate in those digesters. However, the lack of correlation of OTU795 abundance to daily methane production does suggest that the hydrogenotrophic pathway was the dominant methanogenic pathway in this system.

Given that the archaea required for methane production were present based on qPCR data (Figure 3-10F,G), it is unclear whether the lack of methanogenesis in the TSM digesters was due to i) outgrowth of inhibitory microorganisms ii) absence of microorganisms fulfilling compulsory metabolic functions or iii) an inhibitory substrate in the reactors, for example ammonia, suppressing archaeal growth and function (Westerholm et al. 2011). There is also the possibility that while the DNA was detectable, the archaea in the non-functioning digesters were not viable.

It is likely that at pH<6.5 and in the absence of a significant population of acetoclastic methanogens, the most likely mode of methane production in both the TST and TSM reactors was via the syntrophic acetate oxidation pathway, with the resulting H_2 and CO_2 converted to methane by the large population of hydrogenotrophic methanogens (Delbès et al. 2001). Given the persistent acetate accumulation in the TSM digesters, it is possible that the absence of bacteria able to fulfill this metabolic niche under these reactor conditions resulted in poor archaeal methanogenic activity and growth. Quantitative PCR identified two OTU, OTU2686 and OTU2898, both of which correlated negatively to acetate accumulation and positively to daily methane production that could possibly be fulfilling this role. Syntrophic acetate oxidation is not energetically favorable, and is only feasible when coupled with hydrogenotrophic methanogenesis; when H_2 concentrations are high, this reaction is reversed, with bacteria creating acetate from H_2 and CO_2 (Baldrian and Valaskova 2008). The fluctuation between these two methanogenesis pathways is often dependent on environmental and operating conditions. Hydrogenotrophic methanogens tend to be more abundant in thermophilic, low pH, high acetate environments, are more resistant to ammonia

accumulation in the digestate, and are often the most abundant methanogens when these conditions are present in the reactor (Hattori 2008). The lack of acetoclastic methanogens in the TST and TSM reactors and consequent reliance on hydrogenotrophic methanogenesis for methane production may explain their reduced performance compared to INC and MAN reactors, which had significant populations of acetoclastic methanogens able to convert acetate to methane directly, a much more energetically favorable reaction. The reversibility of the hydrogenotrophic reaction may also explain the concurrent methane and acetate accumulation in the TST reactors near the end of the trial period

Further insight into the conditions that are favorable for proliferation of both hydrogenotrophic and acetoclastic methanogens may provide a methanogenic community that is more robust and productive.

3.6 Conclusions

Large-scale shifts were observed in the bacterial community while the archaeal community remained stable. During digestion, the microbial communities shifted towards a common phylogenetic structure, regardless of digester input. Differences in OTU frequency between methane-producing and non-methane-producing reactors provided insight into the bacterial species necessary for acetate catabolization and a successful transition from acidogenesis to methanogenesis. Reactors processing manure contained significant populations of acetoclastic and hydrogenotrophic methanogens, while only hydrogenotrophic methanogens were detected in reactors processing stillage. Quantitative PCR also confirmed the relationship of specific OTU to reactor performance measures including VFA production and catabolization and methane production.

3.7 Acknowledgements

We gratefully acknowledge the support of John Schellenberg and Richard Sparling for the VFA analysis. This work was supported by the ecoENERGY Innovation Initiative (Natural Resources Canada).

CHAPTER 4 - Bioaugmentation relieves acetate accumulation and stimulates methane production in stalled anaerobic digesters

Authors contributions

Conceived and designed the experiments: JRT TJD. Performed the experiments: JRT. Analyzed the data: JRT TJD. Wrote the paper: JRT TJD.

Chapter 4 Preamble

The most common point of failure during anaerobic digestion is the conversion of volatile fatty acids (VFA) to methane, as the archaea and syntrophic acetate-oxidizing bacteria responsible for catabolizing VFA are frequently slower-growing than their acetogenic bacterial counterparts. Left unchecked, the accumulation of VFA in the reactor invariably leads to inhibition of archaeal growth and metabolism, and lack of methane production. Specific microorganisms identified as acetate-catabolizing are logical candidates for bioaugmentation strategies. An *in vitro* culture scheme was developed to isolate and propagate acetoclastic microorganisms identified during the time-course experiment. The resulting consortium, capable of catabolizing acetate to methane *in vitro*, was then evaluated as a bioaugmentation tool for relieving acetate accumulation in stalled reactors.

4.1 Abstract

An imbalance between acidogenic and methanogenic organisms during anaerobic digestion can result in increased accumulation of volatile fatty acids, decreased reactor pH, and inhibition of methane-producing Archaea. Most commonly the result of organic input overload or poor inoculum selection, these microbiological and biochemical changes severely hamper reactor performance, and there are few tools available to facilitate reactor recovery. A small, stable consortium capable of catabolizing acetate and producing methane was propagated *in vitro* and evaluated as a potential bioaugmentation tool for stimulating methanogenesis in acidified reactors. Replicate laboratory-scale batch digesters were seeded with a combination of bioethanol stillage waste and a dairy manure inoculum previously observed to result in high VFA accumulation and reactor failure. Experimental reactors were then amended with the acetoclastic consortium and control reactors were amended with sterile culture media. Within 7 days, bioaugmented reactors had significantly reduced acetate accumulation and the proportion of methane in the biogas increased from 0.2±0% to 74.4±9.9% while control reactors showed no significant reduction in acetate accumulation or increase in methane production. Organisms from the consortium were enumerated using specific quantitative PCR assays to evaluate their growth in the experimental reactors. While the abundance of hydrogenotrophic organisms increased ~10-fold during the experimental period, an acetoclastic methanogen phylogenetically similar to *Methanosarcina* sp. increased more than 100-fold and is hypothesized to be the primary contributor to reactor recovery. Genomic sequencing of this organism revealed genes related to the production of methane from acetate, hydrogen, and methanol.

4.2 Introduction

Anaerobic digestion (AD) of agricultural waste provides an efficient disposal system for organic material while generating renewable energy in the form of methane. Thermophilic AD has been previously shown to be effective for processing stillage waste from corn and wheat-based bioethanol production, both as a single input or co-digested with cattle manure (Agler et al. 2008; Schaefer and Sung 2008; Town et al. 2014a; Westerholm et al. 2012a). Methane generation from organic materials is reliant on the cooperative and interdependent metabolic activities of complex communities of Bacteria and Archaea, the composition and dynamics of which are in many cases poorly understood. Recent efforts to more completely and accurately characterize the microbial communities present in anaerobic digesters have resulted in a more detailed understanding of shifts in the microbial community during digestion, and have identified some of the taxonomic groups responsible for each metabolic stage of methanogenesis (Vanwonterghem et al. 2014a; Werner et al. 2011). The logical next step is to use this phylogenetic and taxonomic information to boost reactor performance, for example by increasing organic loading capacity, reducing hydraulic retention time, or by altering reactor conditions to maximize the growth and metabolic capabilities of the microorganisms present in the reactor. Manipulation of the microbial community to increase reactor efficiency has also been attempted through bioaugmentation, using either complex materials like manure or compost which are rich sources of microorganisms (Schauer-Gimenez et al. 2010; Scherer and Neumann 2013; Westerholm et al. 2012a), or by the addition of exogenous cultures showing desirable metabolic properties *in vitro* (Akila and Chandra 2010; Fotidis et al. 2013; Westerholm et al. 2012b). Generally, the microbiological richness gained by addition of more microbially diverse input has been

consistently shown to be beneficial to reactor performance, improving recovery after toxin exposure or organic overload, and greatly reducing the variability seen when processing some types of waste, including stillage (Schauer-Gimenez et al. 2010; Scherer and Neumann 2013; Westerholm et al. 2012a). Bioaugmentation with cultures of individual organisms or small syntrophic consortia have yielded mixed results, and most of the organisms tried so far are associated with hydrogenotrophic methanogenesis. Reasons for the failure of these consortia to improve reactor performance have mostly been attributed to their inability to thrive in the reactor environment and to out-compete endogenous organisms for nutrients (Fotidis et al. 2013; Westerholm et al. 2012b).

Acetoclastic Archaea, in particular from the genus *Methanosarcina*, have been previously identified as robust methane producers, and have been associated with reactors with consistently elevated levels of acetate (De Vrieze et al. 2012; Lins et al. 2014). Previous genome sequencing efforts for members of this genus have indicated a high level of metabolic diversity, including pathways for acetoclastic, hydrogenotrophic or methanol-catabolizing methanogenesis (De Vrieze et al. 2012). *Methanosarcina* spp. have also been shown to possess genes encoding cytochrome *d* oxidase and superoxide dismutase which are hypothesized to confer aero-tolerance (Maeder et al. 2006). These characteristics suggest that organisms from this taxonomic group would make excellent targets for bioaugmentation and maximization of methane production during anaerobic digestion in cases where organic overload or the accumulation of volatile fatty acids is a concern.

This study describes the successful deployment of an acetate-catabolizing methanogenic consortium to induce methane production in reactors exhibiting symptoms of organic

overload. The effect of the bioaugmentation culture on acetate accumulation and biogas production and composition were analyzed. One consortium member, a *Methanosarcina*-like acetoclastic Archaea which showed very robust growth after deployment was selected for genome sequencing and analysis.

4.3 Materials and Methods

Isolation and culture of bioaugmentation consortium

Minimal media for culturing acetoclastic microorganisms contained 10 mM KH_2PO_4, 25 mM Na_2HPO_4, 10 mM NH_4Cl, 1 mM $MgCl_2 \cdot 6H_2O$, 2 g/L yeast extract, 2 g/L trisodium nitriloacetate, 0.2 g/L $FeCl_3 \cdot 6H_2O$, 0.2 g/L $CoCl_2 \cdot 6H_2O$, 0.1 g/L $MnCl_2 \cdot 4H_2O$, 0.1 g/L $ZnCl_2$, 0.01 g/L $NiCl_2 \cdot 6H_2O$, 0.05 g/L $CaCl_2 \cdot 2H_2O$, 0.05 g/L $CuSO_4 \cdot 2H_2O$, 0.05 g/L $Na_2MoO_4 \cdot 2H_2O$, 2 mM Na_2S and 50 mM sodium acetate. Media was adjusted to pH 7.0 and 10 mL was transferred to Balch tubes fitted with a rubber stopper and secured with a crimped aluminum seal. Media was flushed with N_2 gas twice for 1 minute each and then autoclaved. Prior to inoculation, each culture tube was bled down to 6.89 kPa (1 psi). Balch tubes were inoculated with 100 µl of end-product digestate from a lab-scale digester containing a mixture of thin stillage and dairy cattle manure that had been producing methane for two weeks prior to digestate collection. The consortium was cultivated continuously for 6 months prior to bioaugmentation experiments, and produced consistent levels of biogas *in vitro* as measured with a pressure transducer fitted with a 25 G needle (data not shown). The composition of the consortium was determined by amplification of the type I *cpn*60 and type II thermosome universal targets as described previously (Chaban and Hill 2012; Hill et al. 2006). Amplification products for each target were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). TOP10

cells (Life Technologies, Carlsbad, CA, USA) were transformed with the ligated vector and complete insert sequences were determined for 64 type I and 92 type II clones. Sequences were cleaned and trimmed using LUCY (Chou and Holmes 2001) and assembled into distinct operational taxonomic units (OTU) using CAP3 (Huang and Madan 1999).

Experimental batch reactors

Thin stillage was obtained from Terra Grains (Moose Jaw, SK, Canada), a dry-grind wheat ethanol facility. Total and volatile solids were measured using standard NREL protocols (Table 4-1), and material was stored at -20°C until use. Inoculum was derived by incubating cattle manure from a dairy operation at the University of Saskatchewan (Saskatoon, SK, Canada) anaerobically at 55°C for two weeks and stored at 4°C until use. Inputs were mixed at a 1:1 ratio based on volatile solids content and diluted with sterile water to a total solids concentration of 5%. The input mixture was adjusted to pH 7.1 with calcium hydroxide and 30 mL was added to a 100 mL glass jar, fitted with a silicone septum. Reactors were flushed with N_2 at 82.74 kPa (12 psi) for 5 minutes, bled down to 6.89 kPa (1 psi), and incubated at 55°C in an anaerobic chamber with an atmosphere of 85% N_2, 10% H_2 and 5% CO_2. After 4 weeks, reactors were amended with either 1 mL of bioaugmentation consortium or sterile culture media (n=5). Digestate samples were taken using a wide-bore pipette and stored at -80°C until use.

Table 4-1 The total (TS) and volatile (VS) solids composition of reactor input material.

	%TS	**%VS (of TS)**
Inoculum	12.1	76.5
Thin Stillage	12.0	90.4
Input Mixture	5.02	81.1

Biogas analysis

Total biogas accumulation in the reactors was measured using a pressure transducer (Sper Scientific, Scottsdale, AZ,USA) fitted with a 25G needle. Gas samples were taken weekly and extracted using a 20 mL syringe fitted with a stopcock (Cole Parmer, Vernon Hills, IL, USA) and 25G needle, transferred to a 5 mL evacuated, dehumidified vial, and stored at 4°C until further analysis. Biogas composition was determined using a Varian Micro-GC (CP-2003, Agilent, Santa Clara, CA, USA) equipped with a 10 m Poraplot U column and thermal conductivity detector (TCD). Relative percentages of CO_2, H_2, H_2S and CH_4 were quantified using a 10 m molecular sieve column and TCD with injector and column temperatures of 110°C and 100°C.

Acetate quantification

Total acetate in the digestate samples was measured using a colorimetric acetate quantification kit according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). Briefly, digestate samples were centrifuged at 14,000 *g* for 5 minutes and 10 µl of supernatant was diluted 1/5-1/100 in water. After adding assay buffer and reaction mix to a final volume of 100 µl, reactions were incubated for 40 minutes at room temperature and the absorbance at 450 nm was measured.

Quantitative PCR

Total genomic DNA was isolated from digestate samples using a modified bead beating method described previously (Dumonceaux et al. 2006). Organisms of interest were quantified using OTU-specific qPCR assays as previously described (Town et al. 2014b).

Briefly, each 25 µl reaction included 1x EvaGreen qPCR Master Mix (Biorad, Hercules, CA, USA) and 400 nM of each primer. All primer sequences and PCR conditions are detailed in Table 4-2.

Table 4-2 OTU-specific qPCR primer sequences and amplification conditions.

Target	Primer Sequence (5'-3')	Amplification conditions	Reference
OTU795	CCTAAGCGTTCCCATAGAA TTGCCTCTTCCTGGTCTA	95°C 5 min (1x) 95°C 15 sec; 55°C 20 sec; 72°C 20 sec (40x) 72°C 2 min (1x)	Town et al. Bioresour. Technol. 151:249-257, 2014.
OTU805	AGATCGCCATCGACATCA CTCGGTGAGCTTCTCCTT	95°C 5 min (1x) 95°C 15 sec; 57°C 20 sec; 72°C 20 sec (40x) 72°C 2 min (1x)	Town et al. Bioresour. Technol. 151:249-257, 2014
OTU2923	CCGTCCTGAACGAGCCCTA GCTGCACGACCTTCTCCA	95°C 5 min (1x) 95°C 15 sec; 57°C 20 sec; 72°C 20 sec (40x) 72°C 2 min (1x)	This study
OTU1109	GTCTAACACCATCGGCACTAC CGCTCGCTGTCGGTAATC	95°C 5 min (1x) 95°C 15 sec; 57°C 20 sec; 72°C 20 sec (40x) 72°C 2 min (1x)	This study

Genome sequencing of OTU795

In order selectively enrich for the *Methanosarcina*-like organism associated with OTU795 and to obtain a sample suitable for genome sequencing, the bioaugmentation consortium was treated sequentially with ampicillin (100 mg/L), kanamycin (50 mg/L) and streptomycin (50 mg/L) for 3 weeks each. Every 3-4 days, 5 mL of media was removed and replaced with fresh antibiotic-containing minimal medium as described previously. Following the enrichment process, total genomic DNA was isolated using the Wizard Genomic DNA Extraction Kit (Promega, Madison, WI, USA). The genome was sequenced using a combination of one shotgun sequencing run using Illumina chemistry on a MiSeq sequencer and one 8kb-insert paired end run using Roche454 Titanium Chemistry on a GS Junior sequencer (Hill et al. 2014). The paired end data was assembled using Newbler (v3.0) into scaffolds, and those representing OTU795 were determined based on blastn analysis and GC content. Illumina reads were mapped to the assembled scaffolds using Bowtie2 (Langmead and Salzberg 2012). Mapped Illumina reads for OTU795 were assembled using SOAPdenovo2 (v2.01) (Luo et al. 2012) with kmer size 127 and map length 34. The resulting contigs were then split into 500 bp pieces with a 200 bp overlap using EMBOSS splitter, combined with the paired end reads for OTU795, and re-assembled using Newbler. The sequenced genome was annotated by the Joint Genome Institute (Walnut Creek, CA, USA) and analyzed using the IMG/er portal (Markowitz et al. 2012). The assembled genome sequence can be accessed from GenBank (accession CP011449) or the Joint Genome Institute (taxon identification 2603880174). Individual genes are identified by their JGI Gene ID numbers (http://img.jgi.doe.gov).

4.4 Results and Discussion

Isolation and characterization of the bioaugmentation consortium

The bioaugmentation consortium was isolated and propagated using media and culture conditions similar to those seen in an overloaded reactor in acid crisis. Universal target amplification and sequencing indicated there were 10 type I (*cpn*60) and 3 type II (thermosome) operational taxonomic units (OTU) in the consortium representing at least 9 bacterial and 2 archaeal species (Table 4-3). The detection of Archaea closely related both to *Methanosarcina barkeri* and *Methanoculleus bourgensis* suggested that the consortium has the metabolic capacity for both acetoclastic and hydrogenotrophic methanogenesis. It is also likely that one or more of the Bacteria present in the consortium are acetate oxidizers in a syntrophic relationship with the *Methanoculleus*-like methanogens (Town et al. 2014b). Although the lack of closely related species in the reference database makes it difficult to hypothesize about the specific roles of the detected Bacteria, it does emphasize how the use of culture-independent tools can help to shine a light on previously undiscovered microbes whose culture requirements or reliance on a syntrophic partner for growth have hampered previous characterization efforts. All of the microorganisms detected were 97-100% identical to OTU assembled from the original in-depth characterization of the digestate used to inoculate the culture (Table 4-3) (Town et al. 2014b).

Table 4-3 Frequency of type I *cpn*60 and type II thermosome universal targets in clone library generated from bioaugmentation consortium.

TypeI

Frequency	**Nearest Neighbor cpnDB (%Identity)**	**OTU[1]**
3	*Thermoanaerobacter* sp. (77%)	OTU2886
1	*Clostridium* sp. (77%)	OTU2575
8	*Clostridium* sp. (78%)	OTU2772
2	*Symbiobacterium* sp. (96%)	OTU2923
1	*Desulfotomaculum* sp. (73%)	OTU1109
21	*Methanolinea* sp. (79%)	OTU2750[2]
3	*Pelotomaculum* sp. (75%)	OTU2850
16	*Dethiobacter* sp. (76%)	OTU267201
2	*Desulfotomaculum* sp. (75%)	OTU267204
1	*Ethanoligenens* sp. (77%)	OTU2884

TypeII

Frequency	**Nearest Neighbor cpnDB**	**OTU[1]**
58	*Methanoculleus bourgensis* (β-subunit) (92%)	OTU805[2]
29	*Methanoculleus bourgensis* (α-subunit) (84%)	OTU807[2]
5	*Methanosarcina barkeri* (88%)	OTU795

[1]OTU are identified in a previous study of microbial communities associated with anaerobic digestion of wheat stillage waste (Town et al. 2014b)
[2]genomic sequencing indicated that OTU2750, 805, and 807 derive from the same organism (data not shown)

Induction of reactor failure

Accidental overload of organic material can initiate a sequence of biochemical changes in the reactor that negatively affect methanogenesis and reduce reactor efficiency. A reactor exhibiting these symptoms is characterized by high levels of accumulated volatile fatty acids and low pH, conditions which are known to inhibit the growth of methanogenic Archaea, particularly acetoclastic methanogens (Hattori 2008). The starter inoculum used in this trial was purposefully selected as it had been previously characterized to a great depth using universal target amplification and pyrosequencing (Town et al. 2014b). While abundant populations of hydrogenotrophic methanogens were identified, acetoclastic methanogens were not detected. In previous experiments, this inoculum had also induced high VFA, low pH conditions when combined with thin stillage waste; an ideal situation for assessing the validity of using the methanogenic consortium as a tool for reactor recovery and re-initiation of methanogenesis (Town et al. 2014b). All reactors were seeded with a 1:1 ratio of inoculum and thin stillage based on volatile solids content, and adjusted to a pH 7.1 using calcium hydroxide. By day 28, all ten reactors showed typical characteristics of organic overload and acid crisis (Figure 4-1). Acetate levels increased from 6 mM at day 0 to an average of 84±6.4 mM by day 28 (Figure 4-1), with a corresponding decrease in pH from 7.1 to 6.6±0.23 (Figure 4-2). During the first 28 days of incubation, no methane was detected in the headspace of any of the reactors (Figure 4-1), however the biogas did contain 40.7±4.2% hydrogen gas (data not shown). In the absence of any numerically abundant populations of acetoclastic methanogens, it is likely that this level of hydrogen accumulation inhibited acetate oxidation by syntrophic bacteria and stalling hydrogenotrophic methanogenesis.

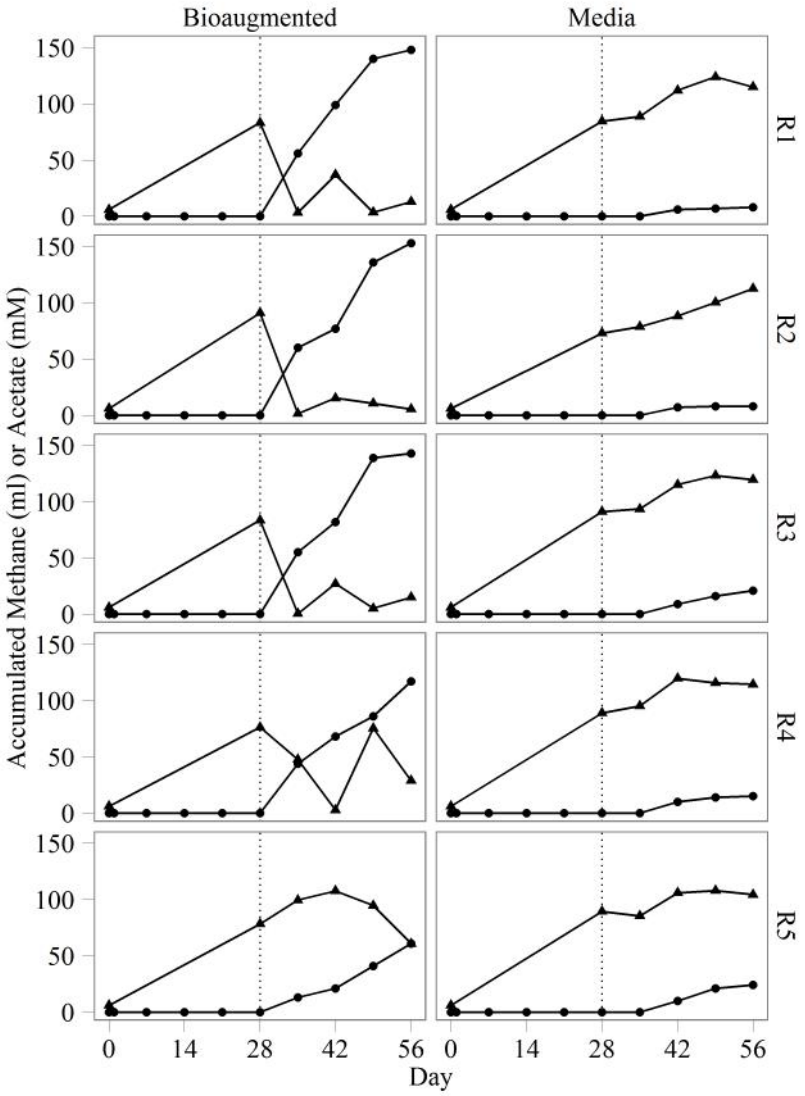


Figure 4-1 Accumulated methane (●) and acetate (▲) in digestate from bioaugmented and media control reactors R1-R5. Digesters were treated with either an acetoclastic consortium or sterile medium on day 28 (indicated by the dotted line).

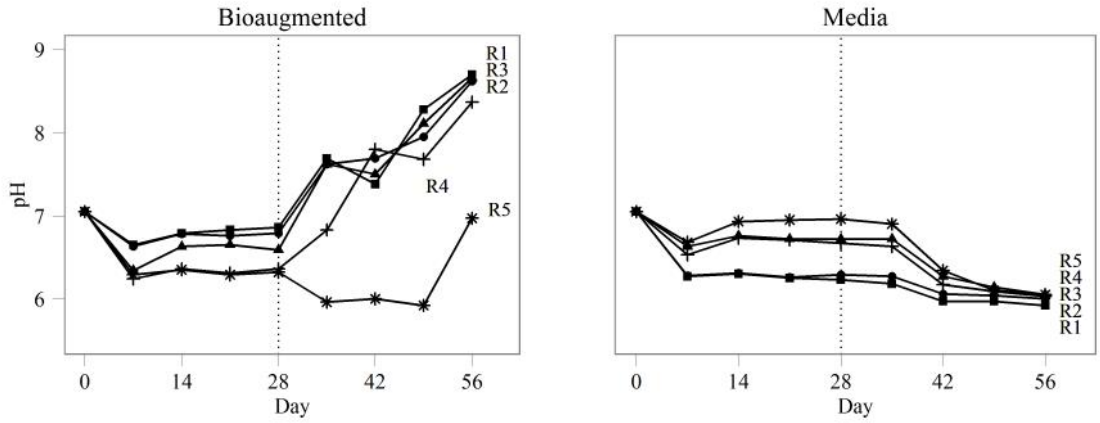


Figure 4-2 Digestate pH in bioaugmented and media control reactors R1-R5. Digesters were treated with either an acetoclastic consortium or sterile medium on day 28 (indicated by the dotted line).

Effect of bioaugmentation on reactor performance

After addition of the acetoclastic consortium, all five bioaugmented reactors showed evidence of methanogenesis within one week while untreated reactors maintained signs of organic overload (Figure 4-1). In 4 of 5 bioaugmented reactors, acetate levels were reduced by 62-94% from peak levels by the end of the experimental period, with a corresponding accumulation of 117-153 mL of methane, indicating that the bioaugmentation consortium was very efficient at catabolizing acetate. These reactors also showed an increase in pH, which is consistent with the removal of acetate and other volatile fatty acids (Figure 4-2). While there was an initial increase in acetate accumulation in reactor 5, acetate catabolization was evident by day 42, and acetate accumulation was reduced 36% by the end of the experimental period and 62 mL of methane produced. In addition to producing higher quantities of methane, biogas produced by reactors that received the acetoclastic consortium contained a higher proportion of methane than control rectors, and this difference was maintained throughout the experimental period (Figure 4-3). Quantification of microorganisms from the bioaugmentation consortium using OTU-specific qPCR assays showed that while some organisms flourished, others showed no significant increase in abundance during the reactor recovery period. In particular, the acetoclastic methanogen OTU795 increased sharply during the experimental period becoming 1.33-250-fold more abundant. In contrast, the abundance of the hydrogenotrophic methanogen OTU805 remained stable during the bioaugmentation period between day 26 and day 58 (Figure 4-4). The lack of proliferation of the Bacteria and the hydrogenotrophic methanogen

suggests that the primary mechanism of recovery was via acetoclastic methanogenesis from the *Methanosarcina*-like Archaea OTU795.

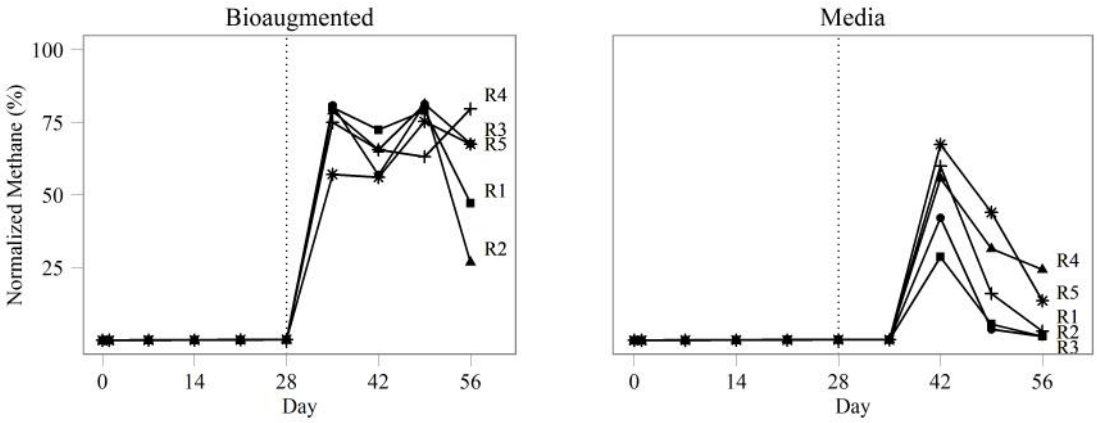


Figure 4-3 Normalized methane as a percentage of total biogas produced in bioaugmented and media control reactors R1-R5. Digesters were treated with either an acetoclastic consortium or sterile medium on day 28 (indicated by the dotted line).

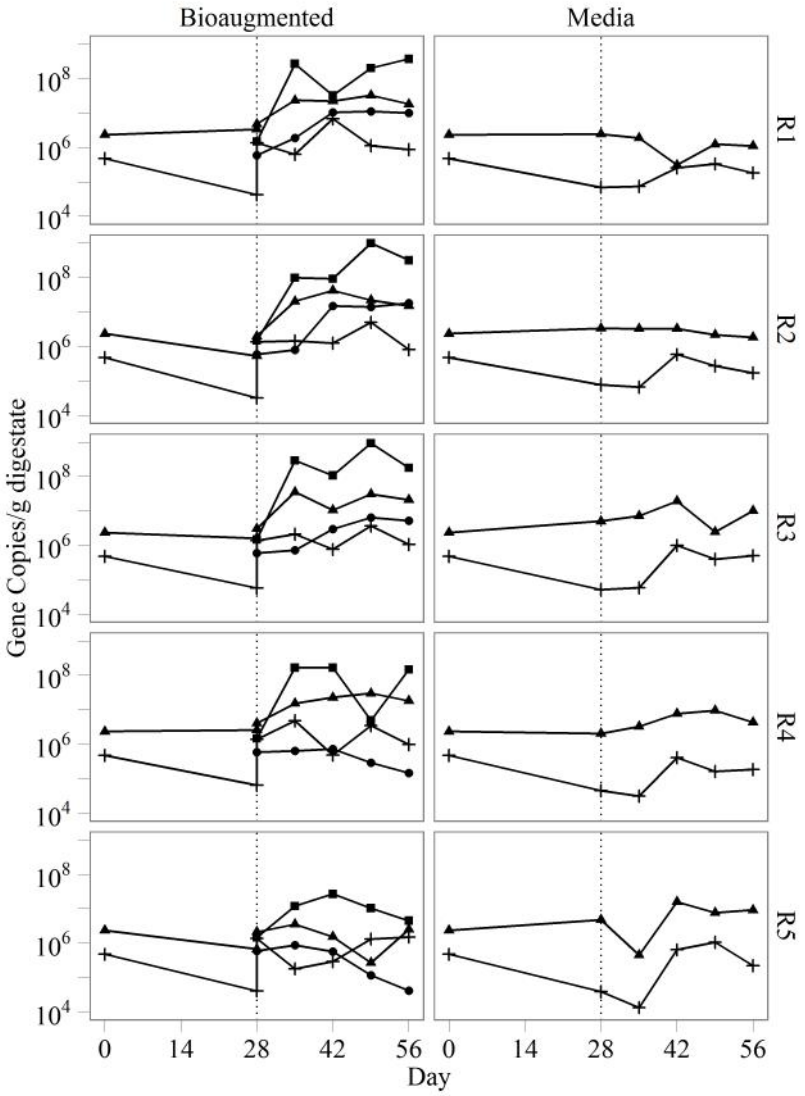


Figure 4-4 Gene abundances of bacterial *cpn*60 for OTU1109 (●) and OTU2923 (▲), and archaeal thermosome for OTU805 (+) and OTU795 (■) in the digestate of bioaugmented and media control reactors R1-R5. OTU2923 and OTU795 were only detected after bioaugmentation treatment. Digesters were treated with either an acetoclastic consortium or sterile medium on day 28 (indicated by the dotted line).

The period of lag time before onset of recovery appeared to be associated with the pH of the digestate at the time of bioaugmentation. *In vitro* characterization of this consortium has indicated that OTU795 grows most vigorously at pH 6.5-8 (data not shown), suggesting the lag time before onset of reactor recovery in bioaugmented reactor R5 was a result of lower digestate pH (6.3) at the time of treatment. This kind of growth information for organisms critical to the digestion process can be beneficial on a larger scale for monitoring and operating digesters at maximum capacity. It can also be useful for identifying ideal growth parameters including temperature, pH and nutrient requirements to maximize the effectiveness of microbial bioaugmentation. Previous studies examining the addition of exogenous cultures in a similar manner have not been as effective either because of an inability of the introduced organisms to colonize effectively (Fotidis et al. 2013), or their inability to reproduce the desired metabolic activity within a complex mixed community (Westerholm et al. 2012b). This consortium was isolated from digestate collected during a successful digestion of stillage products increasing the likelihood that it would thrive within similar operating parameters. While this experiment used batch digester conditions to facilitate the onset of volatile fatty acid overload and monitor the recovery of methanogenesis, it did eliminate the possibility of washout during the initial lag phase post-treatment, allowing the exogenous organisms to become well-established. Washout of slow-growing exogenous cultures has been cited previously as a contributing cause of failure during reactor bioaugmentation (Fotidis et al. 2013) and the success of this biofilm-forming consortium may indicate that immobilization of microorganisms within the reactor may assist slow-growing exogenous

consortia to compete with endogenous microorganisms, particularly in a continuous-flow system.

Unamended reactors did show some biochemical evidence of methanogenesis beginning at days 42-49 suggesting there may have been a small increase in microbial activity stimulated by the addition of the sterile media, however the volume of biogas produced was significantly lower than in the bioaugmented reactors ($p<0.001$) and the effects were transient (Figure 4-2).

Genome sequencing analysis of *Methanosarcina*-like OTU795

The draft genome for this acetoclastic Archaea consisted of one 3.1Mb scaffold composed of nine contigs and a second 2.7 kb scaffold. A summary of general genome features is outlined in Table 4-4.

Table 4-4 Summary of genome features for the acetoclastic methanogen represented by OTU795.

	OTU795
Size (bp)	3,124,993
G+C (%)	41.21
Total genes	2,757
Protein coding genes	2,695
With predicted function	2,084
Without predicted function	611
RNA genes	62
rRNA	6
5S	2
16S	2
23S	2
tRNA	52
Other	4

Comparing average nucleotide identity of OTU795 by BLAST (ANIb), MUMmer (ANIm), and tetranucleotide frequency to sequenced genomes from *Methanosarcina barkeri* Fusaro, *M. acetovorans* C2A, *M.mazei* Tuc01 and *M. mazei* Go1 showed a very low identity among these strains, suggesting that this organism represents a new species within the genus *Methanosarcina* (Table 4-5) based on the species cut-off of 95% identity using JSpecies (Richter and Rossello-Mora 2009).

Table 4-5 Average nucleotide identity between OTU795 and several *Methanosarcina* spp. by blast (ANIb), MUMmer (ANIm), and tetranucleotide frequency.

GenBank accession	JGI Genome ID	vs. OTU795	ANIb	ANIm	Tetranucleotide Frequency
NC_020389	2540341077	*Methanosarcina mazei* Tuc01	76.31%	84.07%	0.935
NC_003901	638154509	*Methanosarcina mazei* Go1	76.75%	84.59%	0.931
NC_007355	637000162	*Methanosarcina barkeri* Fusaro	80.53%	85.22%	0.927
NC_003552	638154508	*Methanosarcina acetivorans* C2A	76.73%	84.07%	0.911

Although substantially smaller in size at only 3.1 Mb compared to 3.4-5.8 Mb for other sequenced *Methanosarcina* spp., OTU795 appears to have similar methanogenic capabilities. Like other M*ethanosarcina* spp., the genome for OTU795 contained all genes required for methanogenic pathways originating from CO_2, acetate and methanol however it lacked the gas vesicle production (*gvp)* operon present in *Methanosarcina barkeri*. While OTU795 contained the majority of the genes required for methane production from formate, unlike *Methanosarcina barkeri*, it did not appear to contain a complete formate dehydrogenase operon encoded by *fdhA* and *fdhB* genes (Maeder et al. 2006). The ability of this organism to catabolize acetate directly likely contributed to its ability to flourish in the stalled reactors, and the ability to utilize multiple methane producing pathways increases its potential value as an indicator of robust reactor performance. Additionally, the two cytochrome *d* oxidase subunits required for oxygen respiration were present (2606038633-34), suggesting that the organism is aero-tolerant, facilitating its propagation and enhancing its resistance to occasional oxygen exposure in the digester environment.

In comparing the number of genes whose predicted function fell into one of the 24 clusters of orthologous groups (COG) categories, it appears that the smaller genome of OTU795 has resulted in fewer genes associated with cell wall biosynthesis, and the transport and metabolism of amino acids and inorganic ions compared to other *Methanosarcina* spp. (Table 4-6). While it also contained fewer genes related to cell motility, OTU795 possessed single copies of the chemotaxis genes *cheABCD* (260603938-39, 260603941-42) and multiple copies of *cheY* (2606040188, 2606040250,

2606040674, 2606040675, 2606040997) encoding for a complete chemotaxis operon (Maeder et al. 2006).

Table 4-6 Comparison of genes assigned to COG categories within the genus *Methanosarcina*.

	Number of genes				
	OTU795	*M. mazei* Tuc01	*M. mazei* Go1	*M. barkeri* Fusaro	*M. acetivorans* C2A
Genome size (Mb)	3.12	3.43	4.1	4.87	5.75
COG description					
RNA processing and modification	1	1	1	1	1
Chromatin structure and dynamics	3	3	3	3	3
Energy production and conversion	178	169	198	210	251
Cell cycle control, cell division, chromosome partitioning	15	14	14	17	16
Amino acid transport and metabolism	157	176	193	220	259
Nucleotide transport and metabolism	65	68	69	71	79
Carbohydrate transport and metabolism	74	66	79	93	107
Coenzyme transport and metabolism	154	158	176	195	243
Lipid transport and metabolism	33	27	32	35	41
Translation, ribosomal structure and biogenesis	190	190	214	218	225
Transcription	75	87	115	112	141
Replication, recombination and repair	71	64	85	80	117
Cell wall/membrane/envelope biogenesis	86	84	117	129	133
Cell motility	11	24	29	20	30
Posttranslational modification, protein turnover, chaperones	92	97	115	124	151
Inorganic ion transport and metabolism	107	121	139	207	255
Secondary metabolites biosynthesis, transport and catabolism	24	23	28	29	44

4.5 Conclusions

The results of this study show that the deployment of an exogenous culture as a bioaugmentation tool to facilitate reactor recovery is feasible on a laboratory scale. The use of digestate from a previously successful reactor as the seed inoculum for the isolation and propagation of the bioaugmentation consortium likely contributed to the ability of these organisms to flourish in the reactor environment. The taxonomic composition of the consortium indicates metabolic diversity with several Bacteria and both acetoclastic and hydrogenotrophic Archaea present, however it is unclear if this community would be able to contribute to a similar digester recovery if there were changes in the input material, nutrient availability, or reactor configuration. In particular, the newly cultured acetoclastic Archaea described here shows excellent potential as a prospective target for digester monitoring using qPCR and inoculum selection. This further highlights how the use of next generation sequencing technologies can lead to the identification and isolation of novel organisms with industrial potential.

While these results are promising, there are challenges with using exogenous bioaugmentation cultures in an industrial setting, due to both larger reactor size and potential differences in reactor configuration. Identifying causal relationships between specific microorganisms and biochemical steps during methanogenesis can identify potential targets for accurate system monitoring, digestate recycling or inoculum screening.

4.6 Acknowledgements

We gratefully acknowledge Dr. Matthew Links for his help with genome assembly and bioinformatics analysis. This work was supported by the ecoENERGY Innovation Initiative (Natural Resources Canada) and Agriculture and Agri-Food Canada.

CHAPTER 5 - Genomic sequencing of OTU1109 and OTU805

Authors contributions

Conceived and designed the experiments: JRT TJD. Performed the experiments: JRT. Analyzed the data: JRT TJD. Wrote the paper: JRT TJD.

Chapter 5 Preamble

Despite considerable effort, it was not possible to obtain a pure isolate of the acetoclastic methanogen OTU795 for genome sequencing. The selective use of antibiotics resulted in a sample suitable for genome sequencing consisting of two archaea and one bacterium. Using a combination of Illumina shotgun and Roche 454 paired end sequencing technology, high quality draft genomes for OTU795 and OTU1109, and a preliminary draft sequence for OTU805, were obtained. Analysis of the OTU795 genome was previously detailed in Chapter 5. While OTU805 and OTU1109 did not seem to be as critical to digester recovery based on their slow growth in the reactors, genome sequencing analysis indicates they likely represent novel species with interesting metabolic capabilities.

5.1 Introduction

The application of next generation sequencing technologies to mixed communities to obtain individual genomes has been very successful. Genome assemblies have been generated for recalcitrant microorganisms from samples including deep ocean sediments and peat bogs (Iverson et al. 2012). To obtain high quality draft genomes using these methods, both technical and bioinformatics considerations must be taken into account. While Illumina sequencing technology provides excellent depth with a very high number of sequencing reads, their short read length (50-300 bp) and small fragment length (500-1000 bp) present sequencing assembly challenges. The addition of Roche Paired End sequencing technology provides longer read length (400-450 bp) and facilitates scaffolding with its use of larger fragments (8 or 20 kb). Other techniques that exploit long fragment generation such as Hi-C contact mapping, have also facilitated de-multiplexing sequencing reads from mixed samples into individual genome assemblies (Burton et al. 2014). Obtaining genome sequences for organisms from extreme environments such as anaerobic digesters provides an opportunity to examine the metabolic capacity of critical microorganisms, discover novel genes and pathways related to methanogenesis, and to potentially develop "designer" organisms to boost digestion efficiency.

5.2 Materials and Methods

Sample preparation

The bioaugmentation consortium outlined in Section 4.3, was subjected to sequential antibiotic selection using ampicillin (100 mg/L), kanamycin (50 mg/L) and streptomycin (100 mg/L). The consortium was maintained in minimal media containing 10 mM

KH_2PO_4, 25 mM Na_2HPO_4, 10 mM NH_4Cl, 1 mM $MgCl_2 \cdot 6H_2O$, 2 g/L yeast extract, 2 g/L trisodium nitriloacetate, 0.2 g/L $FeCl_3 \cdot 6H_2O$, 0.2 g/L $CoCl_2 \cdot 6H_2O$, 0.1 g/L $MnCl_2 \cdot 4H_2O$, 0.1 g/L $ZnCl_2$, 0.01 g/L $NiCl_2 \cdot 6H_2O$, 0.05 g/L $CaCl_2 \cdot 2H_2O$, 0.05 g/L $CuSO_4 \cdot 2H_2O$, 0.05 g/L $Na_2MoO_4 \cdot 2H_2O$, 2 mM Na_2S and 50 mM sodium acetate. Culture material was left to flocculate to the bottom of the tube and 5 ml of culture was removed every 3-4 days and replaced with fresh medium using a 5 ml syringe and 22 G needle. After media exchange, antibiotic was added to 50 mg/L or 100 mg/L as indicated. The culture was treated for 3 weeks with each of ampicillin, kanamycin and streptomycin. Continuous biogas production throughout the selection period was monitored with a pressure transducer fitted with a 25 G needle. Total genomic DNA was purified using the Wizard Genomic DNA Extraction Kit (Promega, Madison, WI, USA). A hydro-shear was used to fragment genomic DNA for Illumina sequencing library preparation using the NEB Next Illumina Prep Kit (NEB, Ipswich, MA, USA), and the library was sequenced using MiSeq chemistry (Illumina, San Diego, CA, USA). The Paired-End library was prepared using a modified Roche protocol as described previously (Hill et al. 2014), and sequenced using Roche GS Junior Titanium Chemistry.

Quantitative PCR

Organisms of interest were quantified using OTU-specific qPCR assays developed using Beacon Designer v 7.0 (Premier Biosoft, Palo Alto, CA, USA). Briefly, each 25 µl reaction included 1x EvaGreen qPCR Master Mix (Biorad, Hercules, CA, USA) and 400 nM of each primer. All primer sequences and PCR reaction conditions are detailed in Table 2-1.

Table 5-1. OTU-specific qPCR primer sequences and amplification conditions.

Primer Sequence (5'-3')	Amplification conditions	Target
CCTAAGCGTTCCCATAGAA TTGCCTCTTCCTGGTCTA	95°C 5 min (1x) 95°C 15 sec; 55°C 20 sec; 72°C 20 sec (40x) 72°C 2 min (1x)	OTU795
AGATCGCCATCGACATCA CTCGGTGAGCTTCTCCTT	95°C 5 min (1x) 95°C 15 sec; 57°C 20 sec; 72°C 20 sec (40x) 72°C 2 min (1x)	OTU805

Genome assembly

The Roche Paired-End data was assembled using Newbler (v3.0) into scaffolds, and those representing OTU1109 were determined based on blastn comparison to all publically available sequences in GenBank and %GC content. Illumina reads were mapped to the assembled scaffolds using Bowtie2 (Langmead and Salzberg 2012). Mapped Illumina reads for OTU1109 were assembled using SOAPdenovo2 (v2.01) (Luo et al. 2012) with kmer size 127 and map length 34. The resulting contigs were then split into 500 bp pieces with a 200 bp overlap using EMBOSS splitter, combined with the paired end reads for OTU1109, and re-assembled using Newbler (v3.0). The draft genome sequence for OTU1109 was annotated by the Joint Genome Institute (Walnut Creek, CA, USA) and analyzed using the IMG/er portal (Markowitz et al. 2012). The assembled genome sequence can be accessed from GenBank (accession LDJB00000000) or the Joint Genome Institute (taxon identification 2603880208). Individual genes are identified by their JGI Gene ID numbers (http://img.jgi.doe.gov).

OTU805 was not abundant enough in the antibiotic-treated consortium to be detected in the Roche paired end sequencing run. As a result, the Illumina reads that did not map to either OTU795 or OTU1109 were assigned as reads originating from the OTU805 genome and were assembled using SOAPdenovo2 with kmer size 127 and map length 34.

5.3 Results and Discussion

Effect of antibiotic selection

The use of antibiotics greatly reduced the number of bacteria present in the consortium as only *cpn*60 sequences from OTU1109, OTU795 and OTU805 were assembled from the

Illumina sequencing data and only OTU795 and OTU1109 were detected in the Roche paired end sequencing data. Additionally, only type II chaperonin sequences from OTU795 and OTU805 were assembled from all sequencing reads. OTU-specific qPCR assays showed that the proportion of OTU795 increased relative to OTU805, likely a direct result of the loss of bacteria that were oxidizing acetate to produce H_2 and CO_2 for hydrogenotrophic methane production (Figure 5-1).

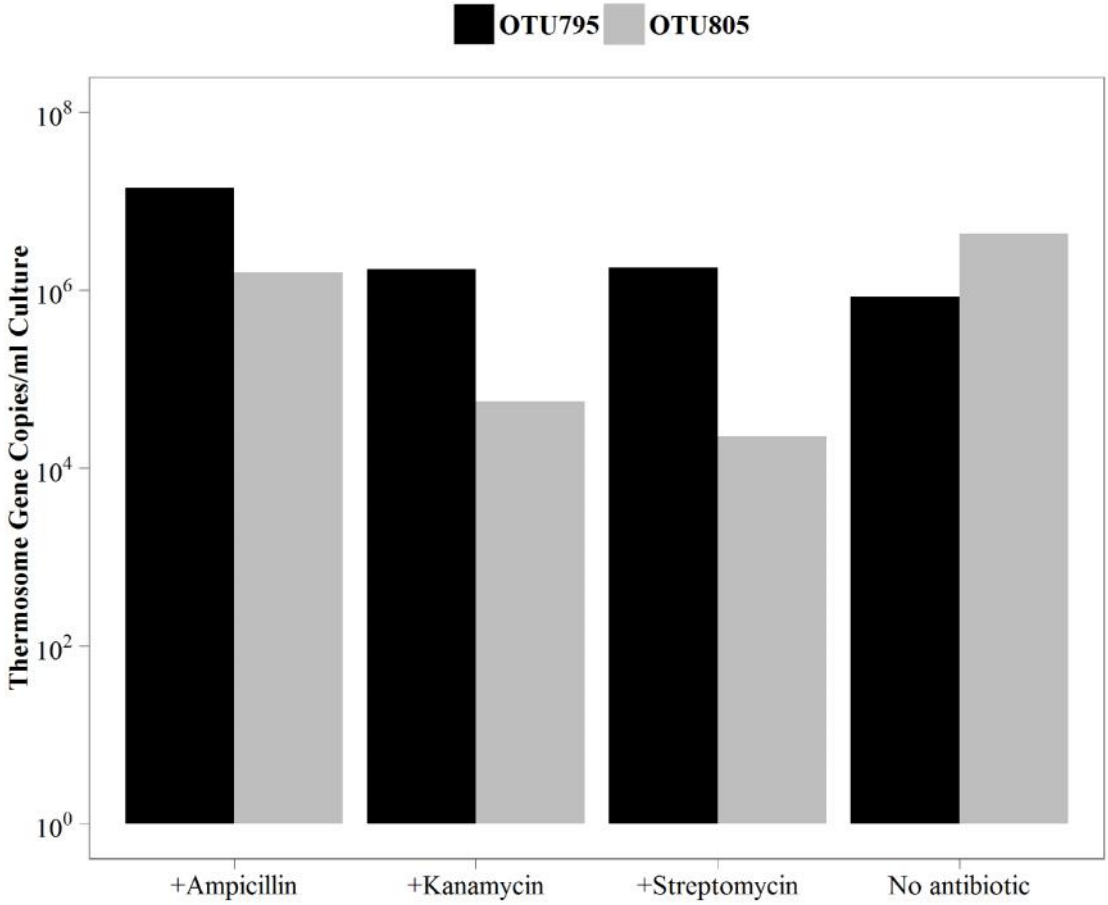


Figure 5-1 Average abundance of OTU795 (*Methanosarcina barkeri*, 88%) and OTU805 (*Methanoculleus bourgensis*, 90%) in the bioaugmentation consortium after treatment with ampicillin (100 mg/L), kanamycin (50 mg/L) and streptomycin (100 mg/L).

Analysis of OTU1109 genome

The final assembly for OTU1109 genome generated 12 scaffolds (N50 = 653,310 bp) composed of 93 contigs and the total genome size was estimated to be 2.7 Mb. Comparing the average nucleotide identity of the OTU1109 genome to sequenced genomes from the most closely related *Desulsufotomaculum* spp. by BLAST (ANIb), MUMmer (ANIm), and tetranucleotide frequency using JSpecies indicated that the organism likely represents a novel species as it is below 95% nucleotide identity threshold set by Richter and Rossello-Mora (Table 5-2) (Richter and Rossello-Mora 2009). Comparison of the amino acid sequences of 40 single copy phylogenetic marker genes from the OTU1109 genome to all publically available prokaryotic genome sequences using specI (Mende et al. 2013) indicated the genome sequence "could not be assigned to a species cluster", suggesting it may represent a novel genus.

Table 5-2 Average nucleotide identity between OTU1109 and several *Desulfotomaculum* spp. by blast (ANIb), MUMmer (ANIm), and tetranucleotide frequency.

GenBank accession	vs. OTU1109	ANIb	ANIm	Tetranucleotide Frequency
NC_015573.1	*Desulfotomaculum kuznetsovii*	63.16%	84.6%	0.553
NC_015565.1	*Desulfotomaculum carboxydivorans*	62.62%	NaN%	0.605
NC_013216.1	*Desulfotomaculum acetoxidans*	62.1%	NaN%	0.561

OTU1109 contained a single copy of the gene encoding formyltetrahydrofolate synthetase (JGI-ID 2606163377), the most common FTHFS biomarker for acetate oxidation (Hori et al. 2011), however no other genes associated with the Wood-Ljungdahl pathway such as CO dehydrogenase, formate dehydrogenase, or hydrogenase were detected (Müller et al. 2013). Additionally, the decrease in relative proportion of the hydrogenotrophic methanogen OTU805 after antibiotic treatment suggests that the role of OTU1109 in the consortium was not syntrophic acetate oxidization. In contrast to OTU795 which contains all genes required for glucose metabolism, OTU1109 contains a full complement of genes for xylose metabolism. This difference in carbohydrate preferences may have contributed to the ability of this organism to flourish in co-culture with OTU795.

The apparent multi-drug resistance seen with this bacterium *in vitro* was likely a result of a combination of genetic and environmental factors. OTU1109 possessed a gene encoding β-lactamase (JGI-ID 2606162626), which likely conferred resistance to ampicillin. While genes associated with aminoglycoside resistance such as kanamycin kinase (JGI-ID 2606161918) and aminoglycoside acetyltransferase (JGI-ID 2606162659) were also present in the OTU1109 genome, it is unclear whether these genes were directly responsible for its resistance to kanamycin and streptomycin treatment (McKay and Wright 1996). Aminoglycosides frequently exploit oxygen-dependent transporters to enter bacterial cells making anaerobic bacteria inherently more resistant (Rasmussen et al. 1997). However, OTU1109 was the only bacterium from the original bioaugmentation consortium to survive antibiotic treatment and so a potential role for these genes cannot be discounted. Additional multi-drug efflux pumps were also

identified, however their function is unknown. Anaerobic digesters have not previously been considered as reservoirs for antibiotic resistance genes however the frequent inclusion of animal waste and the widespread use of antibiotics in the livestock industry suggest this may warrant future consideration.

Analysis of OTU805 genome

As indicated by the quantitative PCR results, the relative proportion of OTU805 was ~100x less than OTU795 and OTU1109 in the antibiotic-treated consortium. As a result, only 4.5% of the Illumina reads originated from this organism, assembling into 2,939 contigs (N50 = 2,037 bp). While a high-quality draft genome assembly was not possible given the limited number of reads, sequencing of the complete *cpn*60 and α and β thermosome subunits for this organism was completed. Blastn analysis of these sequences using all publically available sequence data showed only an 88% nucleotide identity suggesting that this OTU may represent a new species within the genus *Methanoculleus*.

CHAPTER 6 - Conclusions and discussion

6.1 Summary and limitations of these works

There are dynamic changes in microbial community composition during methanogenesis that are consistent among digesters

Metabolic redundancy and niche overlap within complex microbial communities provides the possibility that many definitions of a successful and productive digester community exist. The presence of a core microbial community among productive digesters processing different input material indicates that methanogenic microbial consortia are able to maintain their productivity given a range of input materials. The variations in abundance of phylogenetically similar members of the core microbiome suggest significant niche overlap in the reactors. The time-course study further revealed a shift from a *Clostridium*-dominated bacterial community during hydrolysis to an *Acetivibrio*-dominated community through acidogenesis/acetogenesis, regardless of the combination of input materials.

There was considerably less microbial richness in the archaeal community compared to the bacterial community, however all successful digesters contained robust populations of both hydrogenotrophic and acetoclastic methanogens. The effect of pH on the growth of acetoclastic methanogens, particularly from the genus *Methanosarcina*, was evident during both the time-course and bioaugmentation trials and provides a guideline for required reactor conditions for deploying *Methanosarcina* spp. as bioaugmentation tools.

While the scope of this work did not allow for a statistically robust of the examination of α-diversity measures, there was a substantial increase in community richness and

diversity when manure was added to the input mixture, and manure-amended reactors showed reduced variability in the rate of methane accumulation during the analysis of biochemical methane potential of ethanol production waste streams. The increase in bacterial diversity as a result of manure amendment may also have contributed to the rapid hydrolysis and acidogenesis observed during the time-course trial of thermophilic thin stillage digestion, resulting in high levels of volatile fatty acid accumulation in the inhibition of methanogenic Archaea in manure-amended reactors.

The logical hypothesis going forward is that increased microbial richness provides a greater number of possible microorganisms that would be able to fill any given metabolic niche and would result in more robust digester performance. Further research would be required to determine if diversity can be increased or maintained with a combination of operational conditions and inoculum selection, or if bioaugmentation would be necessary. Even if a significant correlation between α-diversity and reactor performance was established, accurate monitoring of these community traits is complicated and time-consuming using currently available techniques.

Individual microorganisms correlate to reactor performance

Previous studies attempting to establish a link between the composition of the microbial community and digester performance have generated mixed results. While studies have been successful in identifying individual microorganisms whose abundances correlate to digester functions, establishing a direct link between the relative abundances of broader taxonomic groups and digester function has remained elusive (Carballa et al. 2015;

Vanwonterghem et al. 2014b). One of the complications discovered in trying to establish clear correlations is that while dynamic changes in the community have been observed in the short term, there has been increased stability in the long term (Werner et al. 2011). The choice of technique also greatly influences the resolution of the characterization in terms of the taxonomic level of resolution achieved. In situations where niche overlap and redundancy are present, techniques such as universal target sequencing that are capable of species and subspecies resolution may reveal significant changes in the relative abundances of phylogenetically similar organisms, while resolution at the genus or family level as achieved using denaturing gradient gel electrophoresis or restriction fragment length polymorphism, analysis would show no change (Ercolini 2004; Ranjard et al. 2000).

In this study, hydrogenotrophic methanogens were more abundant in all digesters, however those with robust populations of acetoclastic methanogens showed more consistent methane production. In the absence of acetoclastic methanogens, reactors that were also deficient in bacteria identified as potential acetate oxidizers had increased accumulation of volatile fatty acids (VFA) and inhibited methane production. While individual bacteria were identified that correlated to acetate catabolization in the absence of other obvious acetoclastic archaea, an in-depth characterization of these syntrophic acetate oxidizing bacteria (SAOB) and evaluation of their potential as bioaugmentation agents was not within the scope of this study. Isolation and culture of SAOB is very difficult, however previous studies have quantified expression of bacterial formyltetrahydrofolate synthase (FTHFS) genes to confirm acetate-oxidizing activity from specific bacteria (Hori et al. 2011).

This study successfully identified species that were critical for methanogenesis under these conditions, however it remains unclear what level of taxonomic resolution depth of characterization is required to inform operating conditions. While niche overlap may mean that any individual member of a broader taxonomic group may be capable of fulfilling a metabolic role, small differences between members of the same genus may translate to an increased ability of only one particular microorganism to respond to perturbations.

Bioaugmentation is a viable option for digester recovery after acid crisis

Bioaugmentation strategies to improve digester performance have been used for several decades with mixed results. Typically, the bioaugmentation agent used falls into one of two categories: a very rich microbe-laden substrate, most commonly manure or compost, or an exogenous microorganism with a specific and desirable metabolic function. The success of adding complex communities as bioaugmentation agents is suggested to be a function of the resulting increase in microbial richness and diversity. By maintaining and even increasing the total metabolic capacity of the microbiome or increasing the amount of niche redundancy can help communities maintain stable methane production after changes in input material, operating conditions or after toxin exposure. The use of exogenous cultures as bioaugmentation agents have generated mixed results, and generally the failure of these organisms to proliferate in the experimental system has been the primary reason for their lack of effect. This bioaugmentation consortium was isolated from successful digesters processing the same inputs as the failing experimental reactors, likely contributing to its ability to proliferate rapidly in this system. It remains unknown how effective the consortium would be using different substrates or operating conditions.

Additionally, the consortium was filling an empty metabolic niche in the failed digesters, as there was no evidence of acetate catabolization by other organisms in the digester at the time of bioaugmentation, suggesting reduced competition with endogenous microorganisms. The use of a batch reactor system for these experiments also put less reliance on the consortium to maintain the same rapid growth rate as would be required to avoid washout in a continuous flow reactor system. While these factors may have contributed to the success of the consortium in this system, it does leave questions as to whether this strategy would be as successful under different operating conditions or using different input material.

Methanosarcina are uniquely well-suited for maintaining robust methanogenesis

In all experiments outlined in this thesis examining the digestion of stillage and manure, digesters containing the acetoclastic methanogen OTU795, most similar to *Methanosarcina barkeri*, produced more methane at a faster rate than digesters containing only hydrogenotrophic archaea. After addition of an exogenous consortium containing OTU795 to failing reactors, acetate accumulation and inhibition of methanogenesis were alleviated within 7 days. While OTU795 showed the greatest increase in abundance during the bioaugmentation period, this study did not analyze gene expression data and so it is not certain that OTU795 was the most metabolically active organism contributing to digester recovery. Additionally, although significant time and resources were spent trying to obtain a pure culture of OTU795, this was not possible within the time-frame of this study and so examining the growth and metabolism of OTU795 in the absence of other microorganisms was not possible. Sequencing the genome of OTU795 showed it is likely a novel species from the genus *Methanosarcina*

with many of the trademark metabolic capabilities associated with that taxonomic group. Members of the genus *Methanosarcina* have been identified as robust methane producers, uniquely able to perform methanogenesis via acetoclastic, hydrogenotrophic or methylotrophic pathways (De Vrieze et al. 2012). Additionally, the genomes of *Methanosarcina* spp. have been shown to contain multiple nitrogenase, methyltransferase and carbon monoxide dehydrogenase genes making them the most metabolically diverse methanogens (Maeder et al. 2006). While *Methanosarcina* spp. are more susceptible to low pH, high concentrations of volatile fatty acids, and accumulation of ammonia than hydrogenotrophic methanogens, their ability to catabolize acetate directly while not relying on slow growing acetate-oxidizing bacteria gives them the advantage of a faster growth rate (Hattori 2008).

6.2 Discussion of future prospects

Advances in next generation sequencing technologies are facilitating α- and β-diversity comparison on a larger scale

More and more studies are characterizing the microbial community composition and attempting to correlate their numerical abundances or transcript abundance with specific metabolic capabilities. Although research examining β-diversity between samples is rare, there has been some evidence that increases in α-diversity, specifically richness (the number of different organisms) and evenness (distribution of numerical abundances of organisms) has been linked to greater metabolic capacity and redundancy (Werner et al. 2011). These two characteristics would assuredly be beneficial to digester operation, providing metabolic reservoir and increasing the ability of the community to respond more quickly to perturbations (Vanwonterghem et al. 2014a). While continually

monitoring α-diversity in a reactor is considerably more complex than monitoring individual organisms, information gathered from controlled research studies may lead to changes in operation that have the lasting effect of increasing these measures. The application of recently developed Illumina chemistry may facilitate this type of analysis in the future by generating 10-100x more sequencing reads per run than pyrosequencing. Although much of the research in this area is still in its preliminary stages, early indications are that there is definitely an association between α-diversity and process stability during anaerobic digestion (Koch et al. 2014; Werner et al. 2011). While the best methods for achieving and maintaining diversity are not fully known, options may include bioaugmentation with inoculum such as manure or compost to maintain richness, or introducing digestate recycling loops to prevent the washout of slow-growing organisms.

DNA-based assays for microbial quantification are becoming more feasible

The logical next step after identifying organisms that are critical to the digestion process is to develop specific quantitative detection assays and establish benchmark values to correlate their abundances with digester performance. Any method that is suitable for deployment in an industrial setting must be sufficiently sensitive and quantitative, possess a reasonable data collection timeline, and be cost-effective both in terms of required equipment and per-reaction costs. The logistical hurdles faced when trying to culture these organisms suggests that molecular methods are the most logical option for microbial tracking and quantification. The most widely deployed method in a research environment is the polymerase chain reaction (PCR). It is easily customizable for detection at many different taxonomic levels, and cost effective on a per-reaction basis.

However, the expertise required, as well a capital equipment costs, may be a barrier for its widespread adoption in industrial settings. Recently, isothermal loop amplification (LAMP) has been gaining momentum as a suitable molecular diagnostic tool for detecting individual species in a field or industrial setting (Tomita et al. 2008; Tomlinson 2013). Like PCR, LAMP amplifications are directed by species-specific primers, however the amplification occurs at a single temperature which can be maintained using a heat block or water bath. LAMP reactions are also less susceptible to chemical inhibition than PCR, allowing for a more simplistic DNA extraction protocol with fewer purification steps. LAMP results can be obtained in as little as one hour with per reaction costs similar to PCR, but without the need for expensive equipment.

Fluorescence *in situ* hybridization (FISH) is another diagnostic tool that may be practical in an industrial setting (Scherer and Neumann 2013). This technique uses fluorescent DNA probes to detect specific microbial targets. Sample preparation requires minimal equipment and the results are viewed with a properly equipped microscope. While this technique is semi quantitative and may not be suitable for on-going process monitoring, it could provide a quick and cost-effective screening tool for inoculum or bioaugmentation development by ensuring the presence of both hydrogenotrophic and acetoclastic archaea for example.

Modern DNA-based technologies provide an opportunity to generate a complete microbiological and biochemical profile of the digester microbial community. The results of this research suggest that correlative relationships identified using next generation sequencing and quantitative PCR can be used as a basis for determining causal relationships between specific microorganisms and digester performance. The hypothesis

suggested by this work that a well-defined microbial community, with a known composition, metabolic capacity, and defined operating preferences, would be more desirable than a highly variable "black box" community. A well-defined community could be more effectively monitored, and it is possible that with new microbial modelling technologies, its future behavior with known environmental conditions and substrate compositions could be predicted in advance (Larsen et al. 2012).

The transition from a "black box" model to a more defined digestion community would facilitate reactor design and operation

Some of the most difficult aspects of operating a modern anaerobic digester are maintaining continuous, predictable methane production and facilitating digester recovery after a perturbation (Amani et al. 2010). When there is no known information about the community, metadata from the reactor including biogas composition, pH and VFA levels can provide some insight into the root of the problem and the specific biochemical step that has been compromised. The ideal situation however, would be to know exactly which microorganisms were in the digester and their specific metabolic role while simultaneously being able to quantify them in real time. Knowledge of the growth and metabolic properties would allow the operator to modify conditions to increase or reduce the abundance of any particular member. Furthermore, if the consortium was able to be maintained *in vitro* or stored as a bioaugmentation additive, operators could be provided the means to recolonize digesters in the case of a catastrophic event or washout. However, this type of artificial digester colonization would have some foreseeable limitations. It is likely that a well-characterized consortium that has stable growth *in vitro* would have considerably less microbial richness than a typical digester consortium

and potentially a more limited metabolic capacity restricting the diversity of input material that could be effectively digested. Additionally, if the input material was itself a rich source of microorganisms, as is the case with manure, it is unlikely that the original consortium would be able to out-compete the new microorganisms being continuously replenished. However, in situations where the input material is uniform with few endogenous microorganisms, such as bioethanol production waste, brewery waste, and some food waste, this artificial inoculation strategy may provide a means of operating a digester with direct control of the digester microbial community and may provide a level of operating consistency not previously possible.

REFERENCES

Abbasi, T. 2012. Biogas Energy, New York, NY.

Agler, M.T., Garcia, M.L., Lee, E.S., Schlicher, M., and Angenent, L.T. 2008. Thermophilic anaerobic digestion to increase the net energy balance of corn grain ethanol. Environ. Sci. Technol. **42**(17): 6723-6729. doi: 10.1021/es800671a.

Akila, G., and Chandra, T.S. 2010. Stimulation of biomethanation by Clostridium sp. PXYL1 in coculture with a Methanosarcina strain PMET1 at psychrophilic temperatures. J. Appl. Microbiol. **108**(1): 204-213.

Amani, T., Nosrati, M., and Sreekrishnan, T.R. 2010. Anaerobic digestion from the viewpoint of microbiological, chemical, and operational aspects — a review. Environmental Reviews **18**(NA): 255-278. doi: 10.1139/a10-011.

Angelidaki, I. 2009. Defining the biomethane potential (BMP) of solid organic wastes and energy crops : a proposed protocol for batch assays. Wat. Sci. Technol. **59**(5): 927-934.

Angelidaki, I., Alves, M., Bolzonella, D., Borzacconi, L., Campos, J.L., Guwy, A.J., Kalyuzhnyi, S., Jenicek, P., and Van Lier, J.B. 2009. Defining the biomethane potential (BMP) of solid organic wastes and energy crops: A proposed protocol for batch assays.

APHA. 1995. Standard Methods for the Examination of Water and Wastewater 20th Ed.

Balch, W.E., Fox, G.E., Magrum, L.J., Woese, C.R., and Wolfe, R.S. 1979. Methanogens: reevaluation of a unique biological group. MICROBIOL. REV. **43**(2): 260-296.

Baldrian, P., and Valaskova, V. 2008. Degradation of cellulose by basidiomycetous fungi. FEMS Microbiol. Rev. **32**(3): 501-521.

Bond, T., and Templeton, M.R. 2011. History and future of domestic biogas plants in the developing world. Energy for Sustainable Development **15**(4): 347-354. doi: http://dx.doi.org/10.1016/j.esd.2011.09.003.

Bray, J.R., and Curtis, J.T. 1957. An Ordination of the Upland Forest Communities of Southern Wisconsin. Ecological Monographs **27**(4): 326-349. doi: 10.2307/1942268.

Burton, J.N., Liachko, I., Dunham, M.J., and Shendure, J. 2014. Species-level deconvolution of metagenome assemblies with Hi-C-based contact probability maps. G3 Genes Genome Genet. **4**(7): 1339-1346. doi: 10.1534/g3.114.011825.

Canada, E. 2013. National Inventory Report: Greenhouse Gas Sources and Sinks in Canada 1990-2011 The Canadian Government's submission to the UN Framework Convention on Climate Change. doi: http://publications.gc.ca/pub?id=368960&sl=0.

Carballa, M., Regueiro, L., and Lema, J.M. 2015. Microbial management of anaerobic digestion: exploiting the microbiome-functionality nexus. Curr. Opin. Biotechnol. **33**(0): 103-111. doi: http://dx.doi.org/10.1016/j.copbio.2015.01.008.

Carballa, M., Smits, M., Etchebehere, C., Boon, N., and Verstraete, W. 2011. Correlations between molecular and operational parameters in continuous lab-scale anaerobic reactors. Appl. Microbiol. Biotechnol. **89**(2): 303-314. doi: 10.1007/s00253-010-2858-y.

Chaban, B., and Hill, J.E. 2012. A 'universal' type II chaperonin PCR detection system for the investigation of Archaea in complex microbial communities. The ISME journal **6**(2): 430-439.

Chaban, B., Ng, S.Y.M., and Jarrell, K.F. 2006. Archaeal habitats - From the extreme to the ordinary. Can. J. Microbiol. **52**(2): 73-116. doi: 10.1139/w05-147.

Chandra, R., Takeuchi, H., and Hasegawa, T. 2012. Methane production from lignocellulosic agricultural crop wastes: A review in context to second generation of biofuel production. Renewable and Sustainable Energy Reviews **16**(3): 1462-1476. doi: 10.1016/j.rser.2011.11.035.

Chen, Y., Fu, B., Wang, Y., Jiang, Q., and Liu, H. 2012. Reactor performance and bacterial pathogen removal in response to sludge retention time in a mesophilic anaerobic digester treating sewage sludge. Bioresour. Technol. **106**: 20-26. doi: 10.1016/j.biortech.2011.11.093.

Chou, H.-H., and Holmes, M.H. 2001. DNA sequence quality trimming and vector removal. Bioinformatics **17**(12): 1093-1104. doi: 10.1093/bioinformatics/17.12.1093.

Chung, Y.C., Chang, Y.C., Chen, Y.P., Lin, W.C., Lin, H.H., and Tseng, C.P. 2013. Analysis of microbial diversity and optimal conditions for enhanced biogas production from swine waste anaerobic digestion. Journal of Renewable and Sustainable Energy **5**(5). doi: 10.1063/1.4822256.

Dar, S.A., Kleerebezem, R., Stams, A.J.M., Kuenen, J.G., and Muyzer, G. 2008. Competition and coexistence of sulfate-reducing bacteria, acetogens and methanogens in a lab-scale anaerobic bioreactor as affected by changing substrate to sulfate ratio. Appl. Microbiol. Biotechnol. **78**(6): 1045-1055. doi: 10.1007/s00253-008-1391-8.

De Vrieze, J., Gildemyn, S., Vilchez-Vargas, R., Jáuregui, R., Pieper, D., Verstraete, W., and Boon, N. 2015. Inoculum selection is crucial to ensure operational stability in anaerobic digestion. Appl. Microbiol. Biotechnol. **99**(1): 189-199. doi: 10.1007/s00253-014-6046-3.

De Vrieze, J., Hennebel, T., Boon, N., and Verstraete, W. 2012. Methanosarcina: The rediscovered methanogen for heavy duty biomethanation. Bioresour. Technol. **112**: 1-9. doi: 10.1016/j.biortech.2012.02.079.

Delbès, C., Moletta, R., and Godon, J.-J. 2001. Bacterial and archaeal 16S rDNA and 16S rRNA dynamics during an acetate crisis in an anaerobic digestor ecosystem. FEMS Microbiol. Ecol. **35**(1): 19-26. doi: 10.1111/j.1574-6941.2001.tb00784.x.

Demirel, B., and Scherer, P. 2008. The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: a review. Rev. Environ. Sci. Biotechnol. **7**(2): 173-190.

Donoso-Bravo, A., Mailier, J., Martin, C., Rodríguez, J., Aceves-Lara, C.A., and Wouwer, A.V. 2011. Model selection, identification and validation in anaerobic digestion: A review. Wat. Res. **45**(17): 5347-5364. doi: 10.1016/j.watres.2011.08.059.

Dumonceaux, T.J., Hill, J.E., Hemmingsen, S.M., and Van Kessel, A.G. 2006. Characterization of intestinal microbiota and response to dietary virginiamycin supplementation in the broiler chicken. Appl. Environ. Microbiol. **72**(4): 2815-2823.

Ercolini, D. 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. J. Microbiol. Meth. **56**(3): 297-314. doi: http://dx.doi.org/10.1016/j.mimet.2003.11.006.

Eskicioglu, C., Kennedy, K.J., Marin, J., and Strehler, B. 2011. Anaerobic digestion of whole stillage from dry-grind corn ethanol plant under mesophilic and thermophilic conditions. Bioresour. Technol. **102**(2): 1079-1086. doi: 10.1016/j.biortech.2010.08.061.

Fotidis, I.A., Karakashev, D., and Angelidaki, I. 2013. Bioaugmentation with an acetate-oxidising consortium as a tool to tackle ammonia inhibition of anaerobic digestion. Bioresour. Technol. **146**: 57-62. doi: 10.1016/j.biortech.2013.07.041.

Franke-Whittle, I.H., and Insam, H. 2013. Treatment alternatives of slaughterhouse wastes, and their effect on the inactivation of different pathogens: A review. Critical Reviews in Microbiology **39**(2): 139-151. doi: 10.3109/1040841X.2012.694410.

Frigon, J.C., and Guiot, S.R. 2010. Biomethane production from starch and lignocellulosic crops: A comparative review. Biofuels, Bioproducts and Biorefining **4**(4): 447-458. doi: 10.1002/bbb.229.

Gannoun, H., Khelifi, E., Omri, I., Jabari, L., Fardeau, M.L., Bouallagui, H., Godon, J.J., and Hamdi, M. 2013. Microbial monitoring by molecular tools of an upflow anaerobic filter treating abattoir wastewaters. Bioresour. Technol. **142**: 269-277. doi: 10.1016/j.biortech.2013.05.036.

Gihring, T.M., Green, S.J., and Schadt, C.W. 2012. Massively parallel rRNA gene sequencing exacerbates the potential for biased community diversity comparisons due to variable library sizes. Environ. Microbiol. **14**(2): 285-290.

Hattori, S. 2008. Syntrophic acetate-oxidizing microbes in methanogenic environments. Microbes Environ. **23**(2): 118-127.

Hemmingsen, S.M., Woolford, C., van der Vies, S.M., Tilly, K., Dennis, D.T., Georgopoulos, C.P., Hendrix, R.W., and Ellis, R.J. 1988. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. Nature **333**(6171): 330-334.

Hendriks, A.T.W.M., and Zeeman, G. 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour. Technol. **100**(1): 10-18. doi: 10.1016/j.biortech.2008.05.027.

Hill, J., Chaban, B., Town, J., Links, M., and Dumonceaux, T. 2014. Modified paired end rapid library preparation protocol for 454 GS Junior 8 kb library preparation using Covaris g-tubes and BluePippin electrophoresis. Protocol Exchange. Available from http://dx.doi.org/10.1038/protex.2014.028.

Hill, J.E., Penny, S.L., Crowell, K.G., Goh, S.H., and Hemmingsen, S.M. 2004. cpnDB: A Chaperonin Sequence Database. Genome Res. **14**(8): 1669-1675.

Hill, J.E., Town, J.R., and Hemmingsen, S.M. 2005. Improved template representation in *cpn60* polymerase chain reaction (PCR) product libraries generated from complex templates by application of a specific mixture of PCR primers. Environ. Microbiol. **8**(4): 741-746.

Hill, J.E., Town, J.R., and Hemmingsen, S.M. 2006. Improved template representation in *cpn60* polymerase chain reaction (PCR) product libraries generated from complex templates by application of a specific mixture of PCR primers. Environ. Microbiol. **8**(4): 741-746. doi: 10.1111/j.1462-2920.2005.00944.x.

Hill, T.C.J., Walsh, K.A., Harris, J.A., and Moffett, B.F. 2003. Using ecological diversity measures with bacterial communities. FEMS Microbiol. Ecol. **43**(1): 1-11.

Hori, T., Sasaki, D., Haruta, S., Shigematsu, T., Ueno, Y., Ishii, M., and Igarashi, Y. 2011. Detection of active, potentially acetate-oxidizing syntrophs in an anaerobic digester by flux measurement and formyltetrahydrofolate synthetase (FTHFS) expression profiling. Microbiology **157**(7): 1980-1989. doi: 10.1099/mic.0.049189-0.

Huang, X., and Madan, A. 1999. CAP3: A DNA Sequence Assembly Program. Genome Res. **9**(9): 868-877. doi: 10.1101/gr.9.9.868.

Hungate, R.E. 1950. THE ANAEROBIC MESOPHILIC CELLULOLYTIC BACTERIA. Bacteriological Reviews **14**(1): 1-49.

Iverson, V., Morris, R.M., Frazar, C.D., Berthiaume, C.T., Morales, R.L., and Armbrust, E.V. 2012. Untangling genomes from metagenomes: Revealing an uncultured class of marine euryarchaeota. Science **335**(6068): 587-590. doi: 10.1126/science.1212665.

Johnson, E.A., Madia, A., and Demain, A.L. 1981. Chemically defined minimal medium for growth of the anaerobic cellulolytic thermophile Clostridium thermocellum. Appl. Environ. Microbiol. **41**(4): 1060-1062.

Koch, C., Müller, S., Harms, H., and Harnisch, F. 2014. Microbiomes in bioenergy production: From analysis to management. Curr. Opin. Biotechnol. **27**: 65-72. doi: 10.1016/j.copbio.2013.11.006.

Kovács, E., Wirth, R., Maróti, G., Bagi, Z., Nagy, K., Minárovits, J., Rákhely, G., and Kovács, K.L. 2015. Augmented biogas production from protein-rich substrates and associated metagenomic changes. Bioresour. Technol. **178**: 254-261. doi: 10.1016/j.biortech.2014.08.111.

Krause, L., Diaz, N.N., Edwards, R.A., Gartemann, K.H., Kromeke, H., Neuweger, H., Puhler, A., Runte, K.J., Schluter, A., Stoye, J., Szczepanowski, R., Tauch, A., and Goesmann, A. 2008. Taxonomic composition and gene content of a methane-producing microbial community isolated from a biogas reactor. J. Biotechnol. **136**(1-2): 91-101.

Labatut, R.A., and Scott, N.R. 2008. Experimental and predicted methane yields from the anaerobic co-digestion of animal manure with complex organic substrates. *In*, Providence, RI. pp. 6940-6957.

Langmead, B., and Salzberg, S.L. 2012. Fast gapped-read alignment with Bowtie 2. Nature Meth. **9**(4): 357-359.

Lanting, J., and Gross, R.L. 1985. ANAEROBIC PRETREATMENT OF CORN ETHANOL PRODUCTION WASTEWATER. *In*, W Lafayette, IN, USA. pp. 905-914.

Larsen, P.E., Field, D., and Gilbert, J.A. 2012. Predicting bacterial community assemblages using an artificial neural network approach. Nat Meth **9**(6): 621-625. doi: http://www.nature.com/nmeth/journal/v9/n6/abs/nmeth.1975.html#supplementary-information.

Lee, D.H., Zo, Y.G., and Kim, S.J. 1996. Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-strand-conformation polymorphism. Appl. Environ. Microbiol. **62**(9): 3112-3120.

Levén, L., Eriksson, A.R.B., and Schnürer, A. 2007. Effect of process temperature on bacterial and archaeal communities in two methanogenic bioreactors treating organic household waste. FEMS Microbiol. Ecol. **59**(3): 683-693. doi: 10.1111/j.1574-6941.2006.00263.x.

Levin, D.B., Islam, R., Cicek, N., and Sparling, R. 2006. Hydrogen production by Clostridium thermocellum 27405 from cellulosic biomass substrates. Int. J. Hydrogen Energy **31**(11): 1496-1503.

Links, M.G., Chaban, B., Hemmingsen, S.M., Muirhead, K., and Hill, J.E. 2013. mPUMA: a computational approach to metagenomic analysis by de novo assembly of OTUs based on protein-coding barcode sequences. Microbiome **1**(23): in press. doi: 10.1186/2049-2618-1-23.

Links, M.G., Dumonceaux, T.J., Hemmingsen, S.M., and Hill, J.E. 2012. The chaperonin-60 universal target is a barcode for bacteria that enables *de novo* assembly of metagenomic sequence data. PLoS One **7**(11): e49755. doi: 10.1371/journal.pone.0049755.

Lins, P., Reitschuler, C., and Illmer, P. 2014. Methanosarcina spp., the key to relieve the start-up of a thermophilic anaerobic digestion suffering from high acetic acid loads. Bioresour. Technol. **152**: 347-354. doi: 10.1016/j.biortech.2013.11.02010.1128/AEM.01730-13.

Liska, A.J., Yang, H.S., Bremer, V.R., Klopfenstein, T.J., Walters, D.T., Erickson, G.E., and Cassman, K.G. 2009. Improvements in life cycle energy efficiency and greenhouse gas emissions of corn-ethanol. Journal of Industrial Ecology **13**(1): 58-74.

Liu, F.H., Wang, S.B., Zhang, J.S., Zhang, J., Yan, X., Zhou, H.K., Zhao, G.P., and Zhou, Z.H. 2009. The structure of the bacterial and archaeal community in a biogas digester as revealed by denaturing gradient gel electrophoresis and 16S rDNA sequencing analysis. J. Appl. Microbiol. **106**(3): 952-966. doi: 10.1111/j.1365-2672.2008.04064.x.

Lozupone, C., Hamady, M., and Knight, R. 2006. UniFrac--an online tool for comparing microbial community diversity in a phylogenetic context. BMC Bioinformatics **7**: 371.

Lozupone, C., and Knight, R. 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Appl. Environ. Microbiol. **71**(12): 8228-8235.

Luo, G., Xie, L., Zhou, Q., and Angelidaki, I. 2011. Enhancement of bioenergy production from organic wastes by two-stage anaerobic hydrogen and methane production process. Bioresour. Technol. **102**(18): 8700-8706. doi: http://dx.doi.org/10.1016/j.biortech.2011.02.012.

Luo, R., Liu, B., Xie, Y., Li, Z., Huang, W., Yuan, J., He, G., Chen, Y., Pan, Q., Liu, Y., Tang, J., Wu, G., Zhang, H., Shi, Y., Liu, Y., Yu, C., Wang, B., Lu, Y., Han, C., Cheung, D.W., Yiu, S.M., Peng, S., Xiaoqian, Z., Liu, G., Liao, X., Li, Y., Yang, H., Wang, J., Lam, T.W., and Wang, J. 2012. SOAPdenovo2: an empirically improved memory-efficient short-read de novo assembler. GigaScience **1**(1): 18. doi: 10.1186/2047-217x-1-18.

Lv, W., Zhang, W., and Yu, Z. 2013. Evaluation of system performance and microbial communities of a temperature-phased anaerobic digestion system treating dairy manure: Thermophilic digester operated at acidic pH. Bioresour. Technol. **142**: 625-632.

Macias-Corral, M., Samani, Z., Hanson, A., Smith, G., Funk, P., Yu, H., and Longworth, J. 2008. Anaerobic digestion of municipal solid waste and agricultural waste and the effect of co-digestion with dairy cow manure. Bioresour. Technol. **99**(17): 8288-8293. doi: http://dx.doi.org/10.1016/j.biortech.2008.03.057.

Madden, R.H. 1983. Isolation and Characterization of Clostridium stercorarium sp. nov., Cellulolytic Thermophile. Int. J. Sys. Bacteriol. **33**(4): 837-840. doi: 10.1099/00207713-33-4-837.

Maeder, D.L., Anderson, I., Brettin, T.S., Bruce, D.C., Gilna, P., Han, C.S., Lapidus, A., Metcalf, W.W., Saunders, E., Tapia, R., and Sowers, K.R. 2006. The Methanosarcina barkeri genome: Comparative analysis with Methanosarcina acetivorans and Methanosarcina mazei reveals extensive rearrangement within methanosarcinal genomes. J. Bacteriol. **188**(22): 7922-7931. doi: 10.1128/JB.00810-06.

Markowitz, V.M., Chen, I.M.A., Palaniappan, K., Chu, K., Szeto, E., Grechkin, Y., Ratner, A., Jacob, B., Huang, J., Williams, P., Huntemann, M., Anderson, I., Mavromatis, K., Ivanova, N.N., and Kyrpides, N.C. 2012. IMG: the integrated microbial genomes database and comparative analysis system. Nucleic Acids Research **40**(D1): D115-D122. doi: 10.1093/nar/gkr1044.

Massé, D.I., Patni, N.K., Droste, R.L., and Kennedy, K.J. 1996. Operation strategies for psychrophilic anaerobic digestion of swine manure slurry in sequencing batch reactors. Can. J. Civ. Eng. **23**(6): 1285-1294.

Massé, D.I., Talbot, G., and Gilbert, Y. 2011. On farm biogas production: A method to reduce GHG emissions and develop more sustainable livestock operations. Anim. Feed Sci. Technol. **166-167**(0): 436-445. doi: 10.1016/j.anifeedsci.2011.04.075.

McCarty, P.L. 1982. One hundred years of anaerobic treatment. Anaerobic digestion 1981. Proc. symposium, Travemunde: 3-22.

McKay, G.A., and Wright, G.D. 1996. Catalytic mechanism of enterococcal kanamycin kinase (APH(3')-IIIa): Viscosity, thio, and solvent isotope effects support a Theorell-Chance mechanism. Biochemistry **35**(26): 8680-8685. doi: 10.1021/bi9603884.

Mende, D.R., Sunagawa, S., Zeller, G., and Bork, P. 2013. Accurate and universal delineation of prokaryotic species. Nature Meth. **10**(9): 881-884.

Møller, H.B., Sommer, S.G., and Ahring, B.K. 2004. Methane productivity of manure, straw and solid fractions of manure. Biomass Bioenergy **26**(5): 485-495.

Müller, B., Sun, L., and Schnürer, A. 2013. First insights into the syntrophic acetate-oxidizing bacteria – a genetic study. MicrobiologyOpen **2**(1): 35-53. doi: 10.1002/mbo3.50.

Mustafa, A.F., McKinnon, J.J., Ingledew, M.W., and Christensen, D.A. 2000. The nutritive value for ruminants of thin stillage and distillers' grains derived from wheat, rye, triticale and barley. J. Sci. Food Agric. **80**(5): 607-613.

Nasr, N., Elbeshbishy, E., Hafez, H., Nakhla, G., and Hesham El Naggar, M. 2012. Comparative assessment of single-stage and two-stage anaerobic digestion for the

treatment of thin stillage. Bioresour. Technol. **111**: 122-126. doi: 10.1016/j.biortech.2012.02.019.

Nelson, M.C., Morrison, M., Schanbacher, F., and Yu, Z. 2012. Shifts in microbial community structure of granular and liquid biomass in response to changes to infeed and digester design in anaerobic digesters receiving food-processing wastes. Bioresour. Technol. **107**(0): 135-143. doi: http://dx.doi.org/10.1016/j.biortech.2011.12.070.

Nelson, M.C., Morrison, M., and Yu, Z. 2011. A meta-analysis of the microbial diversity observed in anaerobic digesters. Bioresour. Technol. **102**(4): 3730-3739. doi: http://dx.doi.org/10.1016/j.biortech.2010.11.119.

Owen, W.F., Stuckey, D.C., and Healy Jr, J.B. 1979. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. Wat. Res. **13**(6): 485-492.

Pervin, H.M., Dennis, P.G., Lim, H.J., Tyson, G.W., Batstone, D.J., and Bond, P.L. 2013. Drivers of microbial community composition in mesophilic and thermophilic temperature-phased anaerobic digestion pre-treatment reactors. Wat. Res. **47**(19): 7098-7108. doi: 10.1016/j.watres.2013.07.053.

Pullen, T. 2015. Anaerobic Digestion-Making Biogas-Making Energy. Routledge, 711 Third Avenue, New York, NY 10017.

Rabelo, S.C., Carrere, H., Maciel Filho, R., and Costa, A.C. 2011. Production of bioethanol, methane and heat from sugarcane bagasse in a biorefinery concept. Bioresour. Technol. **102**(17): 7887-7895.

Ranjard, L., Poly, F., and Nazaret, S. 2000. Monitoring complex bacterial communities using culture-independent molecular techniques: application to soil environment. Research in Microbiology **151**(3): 167-177. doi: http://dx.doi.org/10.1016/S0923-2508(00)00136-4.

Rasmussen, B.A., Bush, K., and Tally, F.B. 1997. Antimicrobial Resistance in Anaerobes. Clinical Infectious Diseases **24**(Supplement 1): S110-S120. doi: 10.1093/clinids/24.Supplement_1.S110.

Richter, M., and Rossello-Mora, R. 2009. Shifting the genomic gold standard for the prokaryotic species definition. Proc. Natl. Acad. Sci. U.S.A. **106**(45): 19126-19131.

Saha, N.K., Balakrishnan, M., and Batra, V.S. 2005. Improving industrial water use: Case study for an Indian distillery. Resources, Conservation and Recycling **43**(2): 163-174.

Sakar, S., Yetilmezsoy, K., and Kocak, E. 2009. Anaerobic digestion technology in poultry and livestock waste treatment - A literature review. Waste Management and Research **27**(1): 3-18. doi: 10.1177/0734242x07079060.

Sarkar, N., Ghosh, S.K., Bannerjee, S., and Aikat, K. 2012. Bioethanol production from agricultural wastes: An overview. Renewable Energy **37**(1): 19-27. doi: 10.1016/j.renene.2011.06.045.

Sawatdeenarunat, C., Surendra, K.C., Takara, D., Oechsner, H., and Khanal, S.K. 2015. Anaerobic digestion of lignocellulosic biomass: Challenges and opportunities. Bioresour. Technol. **178**: 178-186. doi: 10.1016/j.biortech.2014.09.103.

Schaefer, S.H., and Sung, S. 2008. Retooling the Ethanol Industry: Thermophilic Anaerobic Digestion of Thin Stillage for Methane Production and Pollution Prevention. Wat. Envrion. Res. **80**(2): 101-108. doi: 10.2175/106143007x212157.

Schauer-Gimenez, A.E., Zitomer, D.H., Maki, J.S., and Struble, C.A. 2010. Bioaugmentation for improved recovery of anaerobic digesters after toxicant exposure. Wat. Res. **44**(12): 3555-3564. doi: http://dx.doi.org/10.1016/j.watres.2010.03.037.

Scherer, P., and Neumann, L. 2013. "Methano-compost", a booster and restoring agent for thermophilic anaerobic digestion of energy crops. Biomass Bioenergy **56**(0): 471-478. doi: http://dx.doi.org/10.1016/j.biombioe.2013.05.021.

Schloss, P.D., and Handelsman, J. 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl. Environ. Microbiol. **71**(3): 1501-1506.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., and Weber, C.F. 2009. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. **75**(23): 7537-7541. doi: 10.1128/aem.01541-09.

Sleat, R., Mah, R.A., and Robinson, R. 1984. Isolation and Characterization of an Anaerobic, Cellulolytic Bacterium, Clostridium cellulovorans sp. nov. Appl. Environ. Microbiol. **48**(1): 88-93.

Solli, L., Havelsrud, O., Horn, S., and Rike, A. 2014. A metagenomic study of the microbial communities in four parallel biogas reactors. Biotechnol. Biofuels **7**(1): 146.

Stephenson, M., and Stickland, L.H. 1933. Hydrogenase: The bacterial formation of methane by the reduction of one-carbon compounds by molecular hydrogen. Biochemical Journal **27**(5): 1517-1527.

Suryawanshi, P.C., Chaudhari, A.B., and Kothari, R.M. 2010. Thermophilic anaerobic digestion: The best option for waste treatment. Crit. Rev. Biotechnol. **30**(1): 31-40. doi: 10.3109/07388550903330505.

Thauer, R.K., Jungermann, K., and Decker, K. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriological Reviews **41**(1): 100-180.

Thauer, R.K., Kaster, A.K., Seedorf, H., Buckel, W., and Hedderich, R. 2008. Methanogenic archaea: Ecologically relevant differences in energy conservation. Nat. Rev. Microbiol. **6**(8): 579-591. doi: 10.1038/nrmicro1931.

Tomita, N., Mori, Y., Kanda, H., and Notomi, T. 2008. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat. Protocols **3**(5): 877-882.

Tomlinson, J. 2013. In-field diagnostics using loop-mediated isothermal amplification.

Town, J., Annand, H., Pratt, D., Dumonceaux, T., and Fonstad, T. 2014a. Microbial community composition is consistent across anaerobic digesters processing wheat-based fuel ethanol waste streams. Bioresour. Technol. **157**(0): 127-133. doi: http://dx.doi.org/10.1016/j.biortech.2014.01.074.

Town, J.R., Links, M.G., Fonstad, T.A., and Dumonceaux, T.J. 2014b. Molecular characterization of anaerobic digester microbial communities identifies microorganisms that correlate to reactor performance. Bioresour. Technol. **151**(0): 249-257. doi: http://dx.doi.org/10.1016/j.biortech.2013.10.070.

Vanwonterghem, I., Jensen, P.D., Dennis, P.G., Hugenholtz, P., Rabaey, K., and Tyson, G.W. 2014a. Deterministic processes guide long-term synchronised population dynamics in replicate anaerobic digesters. ISME J **8**(10): 2015-2028. doi: 10.1038/ismej.2014.50.

Vanwonterghem, I., Jensen, P.D., Ho, D.P., Batstone, D.J., and Tyson, G.W. 2014b. Linking microbial community structure, interactions and function in anaerobic digesters using new molecular techniques. Curr. Opin. Biotechnol. **27**(0): 55-64. doi: http://dx.doi.org/10.1016/j.copbio.2013.11.004.

Wang, Y., Zhang, Y., Wang, J., and Meng, L. 2009. Effects of volatile fatty acid concentrations on methane yield and methanogenic bacteria. Biomass Bioenergy **33**(5): 848-853. doi: http://dx.doi.org/10.1016/j.biombioe.2009.01.007.

Ward, A.J., Hobbs, P.J., Holliman, P.J., and Jones, D.L. 2008. Optimisation of the anaerobic digestion of agricultural resources. Bioresour. Technol. **99**(17): 7928-7940. doi: DOI: 10.1016/j.biortech.2008.02.044.

Weiland, P. 2010. Biogas production: Current state and perspectives. Appl. Microbiol. Biotechnol. **85**(4): 849-860. doi: 10.1007/s00253-009-2246-7.

Werner, J.J., Knights, D., Garcia, M.L., Scalfone, N.B., Smith, S., Yarasheski, K., Cummings, T.A., Beers, A.R., Knight, R., and Angenent, L.T. 2011. Bacterial community structures are unique and resilient in full-scale bioenergy systems. Proc. Natl. Acad. Sci. U.S.A. **108**(10): 4158-4163. doi: 10.1073/pnas.1015676108.

Westerholm, M. 2012. Improved biogas production from whole stillage by co - digestion with cattle manure. Bioresour. Technol.

Westerholm, M., Dolfing, J., Sherry, A., Gray, N.D., Head, I.M., and Schnürer, A. 2011. Quantification of syntrophic acetate-oxidizing microbial communities in biogas processes. Environmental Microbiology Reports **3**(4): 500-505. doi: 10.1111/j.1758-2229.2011.00249.x.

Westerholm, M., Hansson, M., and Schnürer, A. 2012a. Improved biogas production from whole stillage by co-digestion with cattle manure. Bioresour. Technol. **114**(0): 314-319. doi: http://dx.doi.org/10.1016/j.biortech.2012.03.005.

Westerholm, M., Levén, L., and Schnürer, A. 2012b. Bioaugmentation of syntrophic acetate-oxidizing culture in biogas reactors exposed to increasing levels of ammonia. Appl. Environ. Microbiol. **78**(21): 7619-7625. doi: 10.1128/AEM.01637-1210.1016/j.jbiosc.2012.05.010.

Woese, C.R., and Fox, G.E. 1977. Phylogenetic structure of the prokaryotic domain: The primary kingdoms. Proc. Natl. Acad. Sci. U.S.A. **74**(11): 5088-5090. doi: 10.1073/pnas.74.11.5088.

Ye, J., Li, D., Sun, Y., Wang, G., Yuan, Z., Zhen, F., and Wang, Y. 2013. Improved biogas production from rice straw by co-digestion with kitchen waste and pig manure. Waste Management.

Young, M.N., Krajmalnik-Brown, R., Liu, W., Doyle, M.L., and Rittmann, B.E. 2013. The role of anaerobic sludge recycle in improving anaerobic digester performance. Bioresour. Technol. **128**: 731-737. doi: 10.1016/j.biortech.2012.11.079.

Zakrzewski, M., Goesmann, A., Jaenicke, S., Jünemann, S., Eikmeyer, F., Szczepanowski, R., Al-Soud, W.A., Sørensen, S., Pühler, A., and Schlüter, A. 2012. Profiling of the metabolically active community from a production-scale biogas plant by means of high-throughput metatranscriptome sequencing. J. Biotechnol. **158**(4): 248-258. doi: http://dx.doi.org/10.1016/j.jbiotec.2012.01.020.

Zhang, C., Mao, Z., Wang, X., Zhang, J., Sun, F., Tang, L., and Zhang, H. 2010. Effective ethanol production by reutilizing waste distillage anaerobic digestion effluent in an integrated fermentation process coupled with both ethanol and methane fermentations. Bioprocess Biosyst Eng **33**(9): 1067-1075. doi: 10.1007/s00449-010-0432-8.

Ziganshin, A., Liebetrau, J., Pröter, J., and Kleinsteuber, S. 2013. Microbial community structure and dynamics during anaerobic digestion of various agricultural waste materials. Appl. Microbiol. Biotechnol. **97**(11): 5161-5174. doi: 10.1007/s00253-013-4867-0.

Ziganshin, A., Schmidt, T., Scholwin, F., Il'inskaya, O., Harms, H., and Kleinsteuber, S. 2011. Bacteria and archaea involved in anaerobic digestion of distillers grains with solubles. Appl. Microbiol. Biotechnol. **89**(6): 2039-2052. doi: 10.1007/s00253-010-2981-9.